UNIVERSITY OF LIVERPOOL

IMMUNO-CHEMOTHERAPEUTIC INTERACTIONS IN BOVINE ONCHOCERCIASIS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

Germanus Soh Bah

July 2018

Summary

Immuno-chemotherapeutic interactions in bovine onchocerciasis

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Onchocerciasis or river blindness is a debilitating skin disease caused by *Onchocerca volvulus,* with 15.5 million people afflicted in Africa. The adult stage of the causative agent, a filarial worm, resides in collagenous nodules and has a lifespan of 10 - 15 years. The control of onchocerciasis using annual mass drug administration with ivermectin targets the first-stage larvae (microfilariae, Mf). However, ivermectin fails to kill adult worms, and the treatment is contraindicated in individuals co-infected with another filarial nematode (*Loa loa*) due to the risk of severe adverse events. Whereas antibiotics of the tetracycline class can be adulticidal, treatment protocols are too long for widespread use. Tetracyclines target the obligate endobacterial symbiont of *O. volvulus* (*Wolbachia*), but the precise mechanism of worm killing is not fully defined. Previous data obtained from a bovine model of onchocerciasis (*Onchocerca ochengi*) indicate that worm death is associated with an immunological shift in the nodule from a neutrophil-dominated response to a local eosinophilia. We attempted to boost eosinophil responses against adult worms by vaccination preceding sub-lethal antibiotic therapy (SLT) in cattle. We assessed for the first time the effect of immunisation against an immunomodulatory antigen, onchocystatin, either alone or in combination with SLT, on skin Mf load, worm viability and intradermal nodule size at six time-points over one year. Systemic antibody and tissue eosinophil responses to vaccination were also evaluated. The effects of oxytetracycline on the female worm proteome and nodular transcriptome were analysed with reference to the *O. ochengi*, *Wolbachia* and bovine genomes. Immunochemotherapy was neither micro- nor macrofilaricidal, although significant levels of serum IgG and nodule eosinophilia were stimulated. “Gold standard” prolonged adulticidal therapy (ADT) induced a 60% reduction in adult worm viability after 52 weeks, while SLT was ineffective. Using label-free proteomics, 50% of 2,548 identified proteins were quantifiable between antibiotic-treated and control female worm samples. *Wolbachia* constituted <1% of these proteins, while the remainder were distributed between *O. ochengi* (30%) and bovine proteins on the worm surface (70%). The majority of the 203 differentially-regulated proteins of bovine origin were significantly enriched for cathelicidin, annexin, serpin, S-100/ICaBP calcium binding, and Ras domains. Those associated with neutrophils were downregulated, whereas an eosinophil major basic protein homologue was upregulated, by the ADT regimen. Worm cuticle proteins were upregulated and all *Wolbachia* proteins were

downregulated by 36 weeks post-treatment. Applying RNA-seq to whole nodules, transcripts were assigned to each organism (68% worm, 28% bovine, 4% *Wolbachia*). In the ADT group, genes regulating apoptosis in bovine mitochondria (VDAC1) and worm neurons (CES-1 and CES-2) were highly expressed at early time-points. Filarial heat-shock proteins and innate immunity receptors were also upregulated, but there was reduced expression of the bovine NKG2D ligand 4 in the ADT group. Conversely, SLT upregulated the production of this ligand and other elements of the bovine immune response. Finally, *Wolbachia* initially upregulated stress-induced morphogen *bolA* and response regulator *pleD* alongside energy metabolism; but the latter was downregulated by the end of the experiment. In conclusion, prior immunisation failed to increase the efficacy of SLT in the *O. ochengi* model. Killing of adult worms following the ADT regimen was associated with decreases in neutrophil antimicrobial proteins, upregulation of an eosinophil granule protein and apoptotic events in both bovine cells and the worm. Disruption of *Wolbachia* energy metabolism and dysregulation of filarial innate immunity may also underlie the fatal effects of antibiotic treatment on *O. ochengi*.

Acknowledgements

I am very grateful to Dr Vincent N. Tanya who recruited me into the filarial research consortium to fill in a gap created by Dr Emmanuel Niba who emigrated to the USA in January 2007. Originally lackadaisical, l accepted the offer, but worked without reserve towards the success of EU projects SCOOTT 2 and 3. I only became interested in filarial research when Dr Benjamin L. Makepeace, my primary supervisor and Dr Vincent N. Tanya, my co-supervisor encouraged me to enrol in a part-time PhD program at the University of Liverpool to be supported with funds from the EU project EPIAF project. Ever since then, my research ambition changed from Veterinary Theriogenology to Parasitology and things have never been the same again. I am also very thankful to Prof. Jonathan M. Wastling, my secondary supervisor, but would have wished to have had more time with him. He facilitated my enrolment into the programme and empowered Ben to guide me throughout this study.

I am very grateful to the University of Liverpool for putting in place an advisory panel to accompany and independently evaluate my research performance. Prof. Diana Williams and Prof. Alan Radford were members of my Independent Progress Assessment Panel. They showed understanding of my problems and advised me on time management, as well as enlightening me on my responsibilities. For this, I am very grateful.

Working in the labs both in the UK and back in Cameroon has been very challenging. Dr Stuart D. Armstrong mentored me on proteomics while Drs Alistair Darby, Dong Xia and Ritesh Krishna assisted and guided me through the processing and quantification of transcriptomics data. For these, l am very grateful to them. The first postdoc researcher I had to share laboratory bench space with at the University of Liverpool was Dr Krystyna Cwiklinski, with whom I attended optional courses on bioinformatics and post genomic technology. She was always keeping an eye on what l was doing and would not hesitate to remind me of the rules while in the laboratory. Consequently, I caused no accident while in the lab; neither was there any serious

contaminations of my samples or our bench space. I am grateful for encouragement received from Paul Gilmore in the Vet. Parasitology and Dr Mariwan Mohammed in the proteomics laboratory. Above all, I am very grateful to the laboratory boss, Catherine Hartley, for providing me with all the safety tips and technical support needed to sail through. She also coordinated the reception and processed all shipment of my samples from Cameroon.

In Cameroon l was supported in the lab by Mme Wachong Kum Ngangyung Hendrietta, Mr Mfopit Youssouf and Mr David Ekale. I am very grateful for their help in the evaluation of worm viability, skin microfilarial count and measurements of nodule diameter. The administrative staff of IRAD Wakwa was led by Dr Messine Ombionyo (2010 – 2013) and late Dr. Abubakar Danjouma (2013 – 2015). They facilitated my fieldwork and endorsed my travel documents. Dr Venasius Lenzemo, the Chief of IRAD Bambui (2015 - 2018) showed a lot of support as l was finalising my studies. Field studies were carried out with the support of Mr Nana Soulemanou (night guard), Mr. Aminou Djida (of blessed memory); the herdsman of the experimental herd. Herdsmen of the IRAD Wakwa Centre, Mr Bobbo Ahmadou, Mr Laminou Mohamadou and Aminou assisted in the tedious exercise of animal restrain during sampling. Mr Hamman Saidou contributed in negotiating the purchase and transportation of the research cattle. Above all, I am particularly grateful to the Director General of IRAD, Dr Woin Noé, for his support towards the completion of this thesis.

My family was in the greatest financial needs while undertaking this study. My junior Brother, Eng. Ernest Forkwa in Nigeria had to miss an academic year because we could not pay his fees while Dr Peter Nji completed his medical course in Accra thanks to support in form of financial loan from one of his university professors. I am grateful to God for enabling them to successfully complete their courses and most importantly for keeping us united as a family thereafter. Without this show of solidarity, particularly on the part of my wife (Mah Nancy Akam); Dad (Mr Bah John Nduh); Mum (Ma Odilia Tifuh Nduh); brothers (Eng. Gerald Ngati Bah and wife Monique, Mr Vitalis Ngwa Bah and wife, Eng., Ernest Forkwa Bah and wife, Mr. Paul

Chick Bah and wife and Dr Peter Nji Bah and wife); sisters (Mrs Mandeng Regina Ngum and husband, Mrs Fonjong Immaculate Anwi and husband and Miss Emmanuella Muyang Bah); and my children (Tifuh Joy Soh, Akohnwi Pedro Akam Soh, Florence-Nightingale Akwi Soh and Amalia Anwi Soh) l could not have made it.

Special thanks go to the Makepeace family (Jenny the wife, and kids Jude and Lois) for the programmed shared meals at the beginning and end of each of my visits to Liverpool. Jenny would come pick me up from my residence and return me to my home at the end. My Cameroonian friends were barrister Gabriel Achunkwe and wife Emeline Mufo; Mme Evelyn Falloh; Michael Ngando alias Locoman; Dr Willy Mbafor and wife Frida; the larger Cameroonian community in Liverpool and Dr Gerald Tata, transporter of extra house luggage back to Cameroon. I am indebted to my Landlord (J Moss Ltd) for the immense support in facilitating the processing of my travel documents and provision of accommodation.

I cannot end this page without listing some of the students with whom l schooled. Drs Patrick Craig, Elsa-Gayle Zekeng, Nicola Beesley, Alison Howell, Walsh Tessa, Lisa Luu and John Graham-Brown, etc.

This study was sponsored by a European Union collaborative research project known by the acronym EPIAF (Enhanced Protective Immunity against Filariasis). I also received financial support from the Bill and Milanda Gates Foundation via the biomarker (BMGF1) project and NIH grants to cover part of my extended study period. Special thanks to the General Manager of IRAD, Dr Noe Woin, for supporting one of my travel expenses to the UK.

Dedication

This work is dedicated to my parents Mr Nduh, Bah John and Madam Nduh, Odilia Tifuh Ngati Bah for the love and mutual understanding.

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List of Abbreviations

Measurements and Parameters

BAM	binary format of Sequence Alignment Map (SAM)
BCS	body condition score
bp	base pairs
°C	degrees Celsius
cm	centimetres
DE	differential expression
FC	fold change
g	centrifugal force
GFF	general feature format
gm	gram
E	east
ELISA	enzyme-linked immunosorbent assay
FDR	false discovery rates
IU	international units
kDa	kiloDalton
L	litre
m	metre
M	molar
Mb	megabases
mg	milligram
Mgf	mascot generic format
ml	millilitre
mm	millimetres
mM	millimolar
ng	nanogram
nM	nanomolar
nm	nanometres
N	north
NEC	normalised *eosinophil* counts

OD	optical density
PCA	principal component analysis
pg	picogram
PP	percent positivity
P	probability
QC	quality control
r	correlation coefficient
RT	room temperature
µg	microgram
µl	microlitre
µm	micrometres
U	unit
V	volts
v/v	volume for volume
w/v	weight for volume
#	number

Organisations, Countries, and Projects

APOC	African Programme for Onchocerciasis Control
CDI	Community-Directed Interventions
CDTI	Community-Directed Treatment with Ivermectin
EPIAF	Enhanced Protective Immunity against Filariasis
ESPEN	Expanded Special Project for Elimination of Neglected tropical diseases
IMF	International Monetary Fund
IRAD	Institute of Agricultural Research for Development
OCP	Onchocerciasis Control Programme
OEPA	Onchocerciasis Elimination Program of the Americas
REMO	Rapid Epidemiological Mapping of Onchocerciasis
UK	United Kingdom
UNDP	United Nations Development Programme
WTSI	Welcome Trust Sanger Institute

WHO	World Health Organisation

Experimental Groups and Time

ADT	Adulticidal oxytetracycline therapy
CON	Untreated control
OVT	Onchocystatin Immunochemotherapy
OVC	Onchocystatin Immunotherapy control
SLT	Sub-lethal oxytetracycline therapy
T0, 4…	Experiment's time point day zero, week four etc
PV	Post-vaccination

Reactions, Reagents and Molecules

ADCC	Antibody dependent cell-mediated cytotoxicity
CAN	Acetonitrile
AEP	Asparagyl endopeptidase
ALT	Abundant Larval Transcript
APC	Antigen presenting cells
BPIP	Bacterial permeability increasing protein
BSA	Bovine serum albumin
CARDS	Caspase recruitment and activation domains CMI Cell-mediated immunity
C3b	Complement fraction 3b
CCL	C-C chemokine ligand
CD-cells	Cluster of differentiated immune cells
cDNA	Complementary deoxyribonucleic acid
CLRs	C-type Lectin receptors
CO_2	Carbon dioxide
CPI	Cysteine Protease Inhibitor
CPI-2	Onchocystatin
CRDs	Carbohydrate recognition domains
CTLDs	C-type lectin domains

CXCR	CXC chemokine receptors
DAB	Diaminobenzidine
DDT	Dithiothreitol
DEC	Diethylcarbamazine-citrate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EDN	Eosinophil-derived neurotoxin
EDTA	Ethylene-diamidine-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	And others
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G-protein coupled signal transduction receptor
GTP	Guanosine triphosphatase
HEPES	N-(2-hydroxyethyl) piperazine-N'-2-(ethanosulphonic acid)
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
ICs	Immune complexes
IFNγ	Interferon-gamma
Ig (G or E)	Immunoglobulin (G or E)
IHC	Immunohistochemistry
IL	Interleukin
IM	Intramuscular
IVM	Ivermectin
LGP2	Laboratory of genetics and physiology 2
LIF	Leucocyte inhibition factor
LPS	Lipopolysaccharides
LRRs	Leucine rich repeats
M1	Macrophages activated by classical routine from monocytes
M2	Alternatively activated macrophages

MBP	Major basic protein
MDA5	Melanoma differentiation-associated gene 5
MHC	Major histocompatibility complex
MMIF	Macrophage migration inhibitory factor
MPO	Myeloperoxidase
MR	Mannose receptors
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
NBD	Nucleotide binding domain
NET	Neutrophil extracellular trap
NF	Nodule fluid
NOD	Nucleotide binding and oligomerisation domain
NLR	NOD-like receptor
*Oo*CPI	*O. ochengi* cysteine protease inhibitor
Ov7	Onchocystatin (*Ov*CPI-2)
PAMPs	Pathogen associated molecular patterns
PAL	Peptidoglycan-associated lipoprotein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K-γ	Phosphatidylinositol 3-kinase gamma
PMN	Polymorphonuclear
PPI	Protein-protein interaction
PRRs	Pathogen recognition receptors
qPCR	Quantitative polymerase chain reaction
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
RIG-1	Retinoic acid-inducible gene 1
RLRs	RIG-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal ribonucleic acid

RLRs	Rig-1-like receptors
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAEs	Severe adverse effects
SHP	Splendore-hoeppli phenomena
SLAP	Secreted larval acidic proteins
TCA	Trichloroacetic acid
TE	10 mM Tris-HCl, 1 mM EDTA, pH 7.5
Tet	Tetracycline
TFA	Trifluoroacetic acid
TLRs	Toll-like receptors
TGF-β	Transforming growth factor-beta
Th	T-helper cells
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor-alpha
Treg	T-regulatory cells
tRNA	Transfer ribonucleic acid
TTBS	'Tween'-20/tris-buffered saline
VAH	Vespid allergen homologue-like protein

Parasites and Disease Conditions

B	*Brugia*
APOD	Acute papular onchodermatitis
CPOD	Chronic papular onchodermatities
F*Oo*WBE	Female *O. ochengi* whole body extract
L3	Third-stage larva
L4	fourth-stage larva
LF	Lymphatic filariasis
Ls	*Litomosoides sigmodontis*
Mf	Microfilariae
Oo	*Onchocerca ochengi*
Ov	*Onchocerca volvulus*

PI	Putative immune
S	*Simulium*
W	*Wuchereria*
WSP	*Wolbachia* surface protein
*w*Bm	*Wolbachia Brugia malayi*
*w*Oo	*Wolbachia Onchocerca ochengi*

Author's Declarations

The following describes the contributions of other individuals to the work presented in this thesis alongside my own.

Chapter 3:

Technicians Henrietta Ngangyung, Youssouf Mouliom Mfopit and David Ekale assisted with parasitological data collection at IRAD Wakwa. In Liverpool, technician Catherine Hartley assisted with optimisation protocol of the ELISA.

Chapter 4:

Dr Stuart Armstrong in Liverpool performed the mass spectrometry on the female worm samples and provided initial training in Progenesis software. He also supplied the methods text for sample preparation and mass spectrometry for this chapter.

Chapter 5:

Dr Pia Koldkjær and other core postdoctoral staff in the Centre for Genomic Research conducted RNA quality control, library preparation and sequencing. Initial processing of RNA-seq data was performed by Dr Alistair Darby in the Centre for Genomic Research. Mapping of the data to the bovine, filarial and symbiont genomes was performed by Dr Ritesh Krishna from the Darby lab. The differential expression and Pfam enrichment analyses were undertaken by Dr Dong Xia from the Wastling lab. Methods text for RNA-seq data processing and analysis was supplied by Drs Krishna and Xia.

I declare that the above is a true and accurate description of how the data presented in this thesis were obtained.

Germanus Soh Bah

Chapter 1 General Introduction

1.1 Background

Onchocerciasis is a vector-borne chronic skin and ocular disease of man caused by *Onchocerca volvulus*, a nematode filarial parasite that cannot replicate itself naturally in any other species, except gorillas (van den Berghe *et al.* 2009). It is a neglected tropical disease endemic in sub Saharan Africa, Latin America, and Yemen. The disease is believed to have originated from West African countries before spreading to the Americas during the slave trade era. Microfilariae (Mf) are responsible for the clinical manifestation of the disease. Reports of the disease have existed for more than two centuries and it was commonly referred to as “craw craw” (meaning itchy, scaly skin) and “river blindness”. Mf were first isolated from skin lesions and described by John O’Neill, a British surgeon on assignment in Sierra Leone (O'Neill 1875). In 1890, the first adult worm was isolated from a nodule sample sent from Ghana and named *Filarial volvulus* by Leuckart, a German scientist. His finding was published by Patrick Manson in 1893, and he renamed the parasite *O. volvulus* (Blacklock 1927). Until 1917, the relationship between Mf and the adult intra-nodular worms was not yet established. Robles in 1917 from Guatamala, South America, provided evidence relating Mf from skin as a larval stage to adult worms located intranodularly, and described the clinical manifestations of the disease. Though he suspected that the black fly could be the vector responsible for the transmission of onchocerciasis, it was Blacklock (1927) who took the credit by proving with experimental evidence that *Simulium damnosum* Theobald, which breeds in fast-

flowing rivers, was the vector transmitting *O. volvulus* (Blacklock 1927). Jean Hissette in 1932 and an American scientist, Strong, in 1938 observed that 20% of onchocerciasis patients on the Sankuru River in Congo were blind and half of the infected cases had visual impairment. Similar correlations between ocular pathology or eye disorders and onchocerciasis were reported in Ghana and Ivory Coast (Prost 1986). Hence, the colloquial "river blindness" became synonymous with onchocerciasis. More eye disorders earlier associated with low skin or plasma vitamin A levels were reassessed and found to be caused by onchocerciasis (Macé *et al.* 1997). *Onchocerca* patients in the early stages of the disease have a high risk of developing night blindness (Babalola 2012).

Ivermectin (IVM) is the drug of choice used in global control programs to eradicate onchocerciasis. The manufacturer of Mectizan® (Merck) donated the drugs free of charge in 1989 for use in mass drug administration in infected areas by the World Health Organisation (WHO) for as long as it takes to eradicate the disease (WHO 2018). The Onchocerciasis Control Programme (OCP), which was launched earlier in 1974 to interrupt disease transmission by vector control in eleven West African countries, ended in 2002 after having freed 18 million children from the risk of blindness and reclaimed more than 250,000 km^2 of fertile land for agriculture (WHO 2018). IVM was introduced in 1987 during the OCP and became the mainstay of the African Programme for the Control of Onchocerciasis (APOC), which extended control to 19 countries outside the OCP. The original goal of APOC was to control onchocerciasis as a public health problem, but it changed its strategy to elimination in 2012 with a target of 80% eradication of onchocerciasis by 2025. The Technical

Consultative Committee of APOC recommended the intensification of current Community-Directed Treatment with Ivermectin (CDTI) and the use of a complementary strategy with alternative Community-Directed interventions CDI (Boussinesq *et al.* 2018), especially in areas with co-infection with *Loa loa* (Wanji *et al.* 2009). Although IVM is not macrofilaricidal, it renders the adult female worms temporarily infertile for at least six months. The WHO estimated with biannual mass drug distribution (MDA) of IVM over fifteen years, equivalent to the longest lifespan of *O. volvulus*, the disease will be eradicated. However, evidence of suboptimal response to IVM treatment has been reported from some foci in Ghana (Osei-Atweneboana *et al.* 2011; Doyle *et al.* 2017) and Cameroon (Nana-Djeunga *et al.* 2014). The main constraint in the control of the onchocerciasis is the lack of flexibility in the choice of drugs available for use.

Antibiotics of the tetracycline family are macrofilaricidal when administered over a long period of time (Langworthy *et al.* 2000; Hoerauf *et al.* 2008b). Considerable efforts have been invested in the screening and identification of safe anthelminthics or antibiotics capable of macrofilaricidal activity with shorter treatment regimens, but to date, only doxycycline has been used in the field, being limited to restricted foci (Taylor *et al.* 2014). Significant insights has been made towards the identification of vaccine candidates (Boursou *et al.* 2018; Lustigman *et al.* 2018) with prophylactic and therapeutic potentials in the laboratory that need to be taken onto the clinical phase (Gregory and Maizels 2008).

One way of diminishing the resistance of an organism is to discover its immune evasive strategy and counteract it. To evade immune attack, filarial parasites secrete

inhibitory molecules that prevent antigen-presenting cells from reporting their presence to T-cells. One major protein involved in worm protection is cysteine protease inhibitor (CPI), or cystatin (Lustigman *et al.* 1992), which interferes with antigen presentation (Manoury *et al.* 2001; Murray *et al.* 2005; Gregory and Maizels 2008) thereby modulating adaptive immunity (Lustigman *et al.* 1991; Babayan *et al.* 2012). CPI-2 from *Onchocerca* species is also known as onchocystatin or Ov7 (Villadangos and Ploegh 2000; Manoury *et al.* 2001). Its presence on the cuticle of adult female worms and most other stages of *O. volvulus*, except mature Mf, makes it a potential vaccine target (Lustigman *et al.* 1991). Onchocystatin also plays an important role in parasite development by arresting the activity of cysteine proteases at the end of each moult (Lustigman *et al.* 1992). Acting as an immune evasion mechanism (Maizels *et al.* 2001), onchocystatin blocks the major histocompatibility complex (MHC) class II site on the antigen presenting cells (APC) and their ability to process antigen via papain-like proteases and asparaginyl endopeptidase (Manoury *et al.* 2001; Murray *et al.* 2005; Kopitar-Jerala 2006; Zavasnik-Bergant 2008; Maizels and McSorley 2016).

Although the macrofilaricidal effect of tetracycline was first discovered some time ago using the cattle *O. ochengi* model (Langworthy *et al.* 2000) and doxycycline confirmed in other *Wolbachia*-harbouring filarial species (Hoerauf *et al.* 2008b), the exact mechanism of worm killing has yet to be clearly elucidated. Experimental evidence from both humans and cattle has shown that worm death is a sequela to depletion of the endosymbiont *Wolbachia*, depopulation of neutrophils and eosinophil degranulation on the worm surface. Eosinophils are effector immune cells

and release cytotoxic granules and mediatory cytokines onto helminths (Pearlman 1997; Pearlman *et al.* 1999). Eosinophils play additional roles in the maintenance of cell integrity and wound healing, and serve as a linkage between the innate and adaptive immune responses (Klion and Nutman 2004; Berek 2016). The release of eosinophils into blood vessels is mediated by IL-5 and / or IL-4 (Horie *et al.* 1996) and they can be activated by a complement-dependent (the preferred site of attachment is on C3b-opsonised parasites) or antibody-dependent (IgG or IgE opsonised parasites) cell-mediated cytotoxicity, leading to a piecemeal degranulation response (Strote *et al.* 1990; Melo and Weller 2010).

To elucidate the mechanism of worm killing by antibiotics, it is essential to understand the basis for mutualism between *Wolbachia* of *Onchocerca spp* and its filarial host. Because *Wolbachia* of *Brugia* (clade D) and that of *Onchocerca* (clade C) belong to different phylogenetic "supergroups", the mutual benefits with their hosts differ. While nutritional dependency on provision of haem and riboflavin by endosymbiont *Wolbachia Brugia malayi* (*w*Bm) may exist (Rao *et al.* 2005), many filariae thrive without *Wolbachia*, including *L. loa (Desjardins et al. 2013)*. Interestingly, uniquely among *Wolbachia*, *Wolbachia Onchocerca ochengi* (*w*Oo) cannot synthesise riboflavin *de novo*; moreover, it cannot convert glucose to pyruvate in common with other *Wolbachia* strains (Darby *et al.* 2012). The worm's glycolytic enzymes seem to interact with *Wolbachia* surface protein to enhance energy production to benefit both parties (S.L. O'Neill *et al.* 2013; Voronin *et al.* 2016). *Wolbachia* may therefore serve as a source of energy for filarial nematodes. It

is most likely that targeting this energy source plays a vital role in worm death after antibiotic therapy.

1.2 Rationale of Study

1.2.1 Immunochemotherapy and Effects on Worm Viability

Cytophilic antibodies (IgG3) generated by putative immune (PI) patients (Boyer *et al.* 1991; Elson *et al.* 1995) target onchocystatin (CPI-2), retard larval development and affect worm viability, with a potential role in the long-term control and prevention of onchocerciasis (Arumugam *et al.* 2014a). Mutation of a key asparagine residue in CPI-2 made the vaccine highly immunogenic (Babayan *et al.* 2012; Arumugam *et al.* 2014b) and using the *Litomosoides sigmodontis* laboratory mouse model, vaccination against mutated onchocystatin (LsCPI) and abundant larval transcript-1 (LsALT) induced significant prophylactic protection when co-administered with Th2 enhancers. We are testing for the first time the combination of vaccination and antibiotic therapy for synergistic effects.

1.2.2 Proteomics and Transcriptomic of Treated Parasites

The proteome and transcriptome of antibiotic-treated *Onchocerca* worms have not been studied before. The concept of a "defence mutualism" between the filarial worm and its endosymbiont has been proposed as the most plausible mechanism underpinning the *Wolbachia* symbiosis with *O. ochengi*. This hypothesis was tested in a study illustrating eosinophil degranulation onto the worm surface before oxytetracycline-mediated death (Hansen *et al.* 2011). Other studies (Frederic Landmann *et al.* 2011) demonstrated that antibiotics cause apoptosis of germline

cells, embryos, and intrauterine Mf. It is essential to give an account of this symbiotic relationship at the transcriptional and protein levels.

1.3 Objectives

The aims of these studies are:

1) To shorten the duration of anti-filarial antibiotic therapy by antibody opsonisation of the worm cuticle, prompting eosinophil degranulation.
2) To identify molecular processes that can better explain the mechanism of worm killing using female worm proteomics and whole-nodule transcriptomics.
3) To identify regulatory processes associated with worm death or survival after antibiotic therapy, paying attention to the worm-*Wolbachia* mutualism.

Chapter 2 Literature Review

2.1 Characteristics of Filarial Nematodes

2.1.1 Origin

Filarial nematodes are parasitic roundworms (**Figure 2.1**) of vertebrates classified in the *Onchocercidae* with 80 genera as members (Anderson 1999). Only five of these genera (*Brugia*, *Wuchereria*, *Onchocerca, Loa* and *Mansonella*) commonly infect humans. The Onchocercidae probably originated from the Oswaldofilariinae of crocodiles that existed 150 million years ago (Chabaud and Bain 1994).

2.1.2 Morphology

A typical nematode has an anterior or cephalic blunt end, a cylindrical, slender and long body, and a tail that narrows to a pointed tip (Cross 1996; El-Massry and Derbala 2000). Worm size varies with sex, age and species. John O'Neill was the first person to describe microfilaria (Mf), the first stage larva (L1) found in the skin of an *O. volvulus* infected person (O'Neill 1875; Neafie 1972). Mf is the motile stage that is released into the skin or blood for onward transmission by a blood-sucking vector. The first person to describe an adult *O. volvulus* was Leuckhart in an unpublished personal communication to Patrick Manson in 1886. A young male *Onchocerca* filaria will attain an adult size of 23 – 40 x 15 mm and female up to 500 – 700 mm long (Neafie 1972; Cross 1996; CAS 2012). A complete description of the anatomy of *O. volvulus* (Neafie 1972) highlighted key features which could be used to distinguish the different human filariae. This report also gave an apt description of the fine structures of the male and female worms, covering the cuticle, reproductive organs and digestive tracts.

Filarial nematodes are pseudocoelomates, with the reproductive and digestive tracts suspended within the pseudocoel. The body wall is composed of the hypodermis, epidermis and cuticle (the latter is outermost) (Franz 1982; Franz and Buttner 1983). The epidermis is a mass of membrane-less cells layered over a pair of longitudinal muscles which secrete the cuticle (Deas *et al.* 1974). The nervous system separates the epidermis from longitudinal muscles dorsally and ventrally. Unlike in the vertebrates, muscle cells branch towards the nerves. There are a series of nerve centres along the ventral nerves and close to the head, and a nerve ring connects the ventral to the dorsal nerves. Structural modifications exist on the cuticle of nodule-forming worms, suggesting adaptation to permit limited active (energy-dependent) processes within the cuticle, allowing selective intake or excretion of substances across the cuticle (Deas *et al.* 1974). The cuticle of the male *Onchocerca* worm is thicker at the lateral regions and contains more layers than at the dorsal and ventral regions than those of the females (Franz 1982). The cuticle is about 90 µm thick, uniformly subdivided into three layers (Deas *et al.* 1974). The porous fibrous layer invaginates into the hypodermal plasmalemma or laminae. External to the fibrous layer is the matrix composed of amorphous material, which could only allow substances through via diffusion, and the cortex covered by an outward-folded trilaminate membrane covered with glycocalyx (Deas *et al.* 1974). The digestive tract of *O. volvulus* consists of a mouth, cuticle-lined oesophagus, and an intestine lined by a single layer of epithelium connected to the anus by a duct or cloaca in males, or cuticle-lined rectum in females (Neafie 1972). The anus terminates 65 or 210 µm from the posterior end of males or females, respectively.

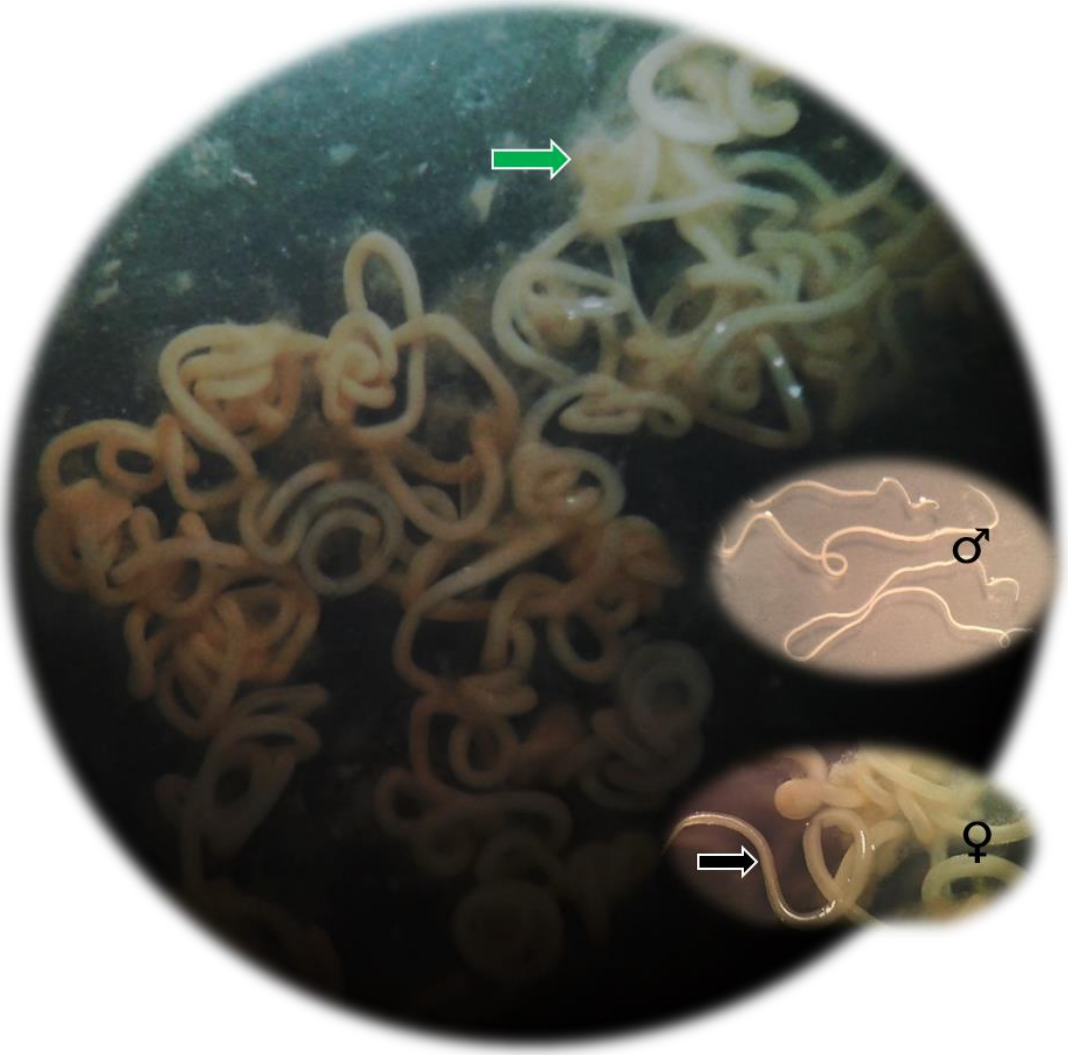


Figure 2.1: Collagenase-digested *O. ochengi* nodule content (original magnification, x10) illustrating the female (♀) worm head (dark arrow) and male worms (♂). Host cells are bound to the coils of the female (green arrow). Image was taken using personal SAMSUNG GT-S5830i phone.

2.1.3 Habitat

Adult filarial parasites dwell in body tissues of vertebrates, the most common sites being the dermis and the lymph vessels. The nodules containing female *O. volvulus* are in the subcutis, while those of its closest relative*, O. ochengi* in cattle are intradermal. Nodules are richly supplied with blood vessels and are the principal source of nutrients to the worm (George *et al.* 1985; Smith *et al.* 1988). There is

evidence for the presence of whole red blood cells in the gut of *Litomosoides sigmodontis* (Attout *et al.* 2005) implying that the worms from time to time consume a blood meal. Lymphatic filariae reside in lymph vessels and resist being carried away to the lymph nodes by adhering to the lymph vessel endothelium or “swimming” in an anti-current direction (Bain and Babayan 2003).

2.1.4 Reproduction and Life Cycle

Filarial worms are dioecious species. The males are mobile, migrating from nodule to nodule to inseminate mature females and are not always present in all nodules. Female worms are sedentary and confined within a nodule (Bwangamoi 1969). The first reports of the reproductive performance of male filariae described the process of spermatogenesis in both *O. volvulus* (Duke *et al.* 1990) and *Wuchereria bancrofti* (Miller 1966). A haploid chromosome number of five was identified in the spermatids of both species. The male reproductive system is a straight tube composed of the testis, vas deferens, and ejaculatory duct, which unites with the intestine to form the cloaca and a pair of spicules for copulation (Neafie 1972). Meanwhile, the female reproduction system consists of paired ovaries, oviducts, seminal receptacle and uteri that unite to form the vagina and ends at the vulva located about 940 µm from the anterior end (Neafie 1972). Sperm once deposited into the vagina becomes amoeboid and migrates into the uteri to fertilise the oocytes. Female worms attain maturity in nine to twelve months. Oogenesis and release of ova into the reproductive tract occur irrespective of the presence of male worms (Schulz-Key and Karam 1986; Schulz-Key and Soboslay 1994). Although there is a strong correlation between the presence of males and fertilisation, there are exceptions where the

males fertilise the female and then migrate out of the nodule, or unfertilised females being present in a nodule with a male (Duke *et al.* 1990). Approximately 1,000 Mf are released into the host's body tissues every day (Schulz-Key 1990; Schulz-Key and Soboslay 1994).

Filarial worms grow by periodically shedding their cuticles during moulting before elongation. During the six months to two-year lifespan of Mf, during which they are either ingested by a vector or eventually die, there is no longitudinal growth. The first two moults occur within the blood sucking arthropod as they penetrate the intestinal tissue and migrate to the salivary glands via the thorax to become the infective L3. During subsequent blood meals, the L3 are deposited into the vertebrate host to infect them. The third and the fourth moults occur within the definitive host. Organogenesis takes place during the development of the larval phase and ends with sexual differentiation during the 4^{th} moult (Bain and Babayan 2003). The basic nutritional needs of the worms are not well known, but it is obvious that infection causes malnutrition in the host, while malnourished patients are much more susceptible to the disease (Storey 1993).

2.1.5 Feeding and Locomotion

The feeding habits of the larvae and worms have not yet been well documented. In *L. sigmodontis*, ingestion of blood occurs only after the fourth moult (Attout *et al.* 2005). The nodules in onchocerciasis have been shown to be richly supplied with blood vessels (George *et al.* 1985), and capillaries are in close apposition with the worm's cuticle (Smith *et al.* 1988). Parasite growth and embryogenesis are retarded when the host is malnourished (Storey 1993). Morphological adaptation and

histological evidence of the cuticle of *O. volvulus* showing mitochondria close to the hypodermal invaginations suggest that there may be trafficking substances across the cuticle (Deas *et al.* 1974).

Filarial worms lack circular muscles and cannot creep. However, they have longitudinal muscles that drive lateral movements. On ingestion of Mf by the vector, they migrate and moult through a series of steps from the gut through the thorax to the mouth of the arthropod vector. In this process, the larvae develop features and behavioural patterns that will facilitate their rapid escape from the skin, when deposited during blood meals, to the safety of the nearest lymphatic vessel (Bain and Babayan 2003). The principal function of the locomotive system is to keep the worm from danger and carry the larvae to locations that are safe for the completion of the worm's life cycle. Contractions of the longitudinal muscles enable adult worms of non-nodule forming species to actively maintain themselves in position within the lymph vessel against the slow flow of lymph current (Dreyer *et al.* 1999). In nodule-forming filariae, the female worms are sedentary and have highly hypotrophied musculature. A greater portion of the female is restrained by fibrous tissues, while the anterior end is free and highly motile. This may be to permit feeding and copulation (Schulz-Key and Karam 1986).

Though filarial worms have a poorly-developed locomotive system, they have developed certain adaptive behaviours to survive (Bain and Babayan 2003). The Mf are motile and can survive in the host tissues for many months, waiting to be consumed by an intermediate host. Some filarial nematodes when gravid, release Mf periodically at different times of the day and night (nocturnally sub-periodic or

periodic) or throughout the day (diurnally sub-periodic or periodic) (McFadzean and Hawking 1956; Duke 1957; Hawking *et al.* 1964; Hawking *et al.* 1965; Nanduri and Kazura 1989). The periodicity of the presence of Mf in the blood or skin of the host may coincide with a rest period such as during deep sleep at night, but is also linked to vector biting behaviour (Nanduri and Kazura 1989).

In the rodent parasite *Acanthocheilonema vitae*, Mf use hooks situated at its anterior end to migrate to the point of release into saliva during blood meals (Bain and Babayan 2003). Sensory organs known as amphids located at the anterior end of the worm might detect changes in the partial pressures of carbon dioxide of surrounding fluid, which is thought to be essential in directing the Mf to detach or attach to nearby tissue (Hawking *et al.* 1965). These sensory and locomotive activities are important factors for the worm's survival that Bain and Babayan (2003) have suggested could be exploited in the control of filarial diseases.

2.1.6 Nodule Formation

Only *Onchocerca* spp. from the five human filarial genera forms nodules. Lack of understanding of the mechanism of nodule production is the main limiting factor to the *in vitro* development of *O. volvulus* for research purposes. It is probable that nodule formation is provoked during the early period of growth of the young worm while provoking a haemorrhage to obtain blood meal. The worm expands to block the thin dermal or sub-dermal lymph vessels (Bain and Babayan 2003). The whole worm is submerged in a highly vascularized fibrin-lake (George *et al.* 1985; Attout *et al.* 2005) which probably contributes to immobilise the posterior part of the female as the condition becomes chronic. As the worm grows and the nodule develops, the

active anterior part of the body becomes avascularised (Figure 2.1), the fibrin lake becomes gel-like and the worms tend to control proliferation of blood vessels (Smith *et al.* 1988). Cell mediated immunity may also contribute to nodule formation as neutrophil extracellular traps (NET) induced by the endosymbiont bacteria *Wolbachia* are formed around the worm (Tamarozzi *et al.* 2016).

2.2 Filarial Diseases

2.2.1 Filarial Diseases of Domestic Animals

Relatively little attention has been given to filarial diseases of domestic animals. The most widely studied disease of animals is dirofilariasis, especially canine heartworm disease caused by *Dirofilaria immitis* (Rani *et al.* 2010). This is the most important filarial disease of dogs transmitted by about 60 species of mosquitoes and can also cause respiratory symptoms in cats. Several other wild carnivores can act as reservoirs. Rarely, humans in exposed localities may also become infected but constitute a dead end for the lifecycle (Haddock 1987; Bowman and Atkins 2009; Lee *et al.* 2010; Ledesma and Harrington 2011). The main horse filarial disease is equine parafilariosis, summer bleeding or bloody sweat (Maloufi 1995). In addition to humans, some strains of *Brugia malayi* infect domestic cats, dogs and monkeys (Rani *et al.* 2010; Ambily *et al.* 2011); while *Cercopithifilaria* sp. (Otranto *et al.* 2012a) and *Onchocerca lupi* (Otranto *et al.* 2012b) infect dogs, with the latter producing ocular disease (Sréter and Széll 2008; Otranto *et al.* 2012b). Given the close relationship that man has with these pet animals, these revelations not only show how filarial diseases of animals have been neglected, but also the danger posed to human health (Uni *et*

al. 2010) and the need to factor animal filariosis in future control programs of human filarioses (Rani *et al.* 2010).

African zebu cattle are host to several *Onchocerca* spp.: *O. ochengi*, *O. gutturosa*, *O. armillata* and *O. dukei* (Wahl *et al.* 1994). The presence of nodules on cattle infected with *O. ochengi* is of little veterinary importance even though hide quality from infected cattle may be reduced (Bwangamoi 1969). Meat condemnations due to bovine onchocerciasis are rare and if done as in Finland, are purely for aesthetic reasons (Solismaa *et al.* 2008) as there is no risk of human infection from consumption of infected meat or hide. Cattle infected with *O. ochengi* are valued as a research model for human *O. volvulus* (Trees *et al.* 2000). Another filaria of cattle is *Setaria* spp., transmitted by *Aedes* mosquitoes. There are a number of species worldwide, some of which are also found in horses, sheep (Tschuor *et al.* 2006), goats and donkeys (Vos *et al.* 1985). The main clinical disorders caused by *Setaria* spp. are nervous symptoms and peritonitis, but generally they are of very little veterinary importance. However, an outbreak of *Setaria* peritonitis was reported in Finnish reindeer (Laaksonen *et al.* 2009).

2.2.2 Filarial Diseases of Man

There are three main types of human filariases: lymphatic filariasis, loiasis and onchocerciasis.

2.2.2.1 *General Characteristics*

The first clinical manifestation of any active human filarial disease is persistent intensive skin irritation associated with vector bite and in onchocerciasis with the release of large numbers of Mf into the skin (Nanduri and Kazura 1989; Murdoch *et*

al. 1993). However, exposure to fly bites, which is often associated with deposition of infective larvae or immunogens, can cause transient pruritus. The first appearance of gross and chronic pathology which differentiates the filarial infections may take several months or years to appear (Storey 1993). Chronic symptoms are associated with wasting, debilitation and disfigurement (Murdoch *et al.* 1993; Enk 2006; Udall 2007) and such patients are stigmatised with severe life threatening consequences (Ovuga *et al.* 1995). Global disease control measures put in place over the past 30 years has significantly reduced these symptoms (Remme *et al.* 2006; Prichard *et al.* 2012).

Human filariases are neglected tropical diseases because they are diseases of the poor (Murdoch *et al.* 1993) and at one time, more than 150 million people in Africa were at risk of infection (Knopp *et al.* 2012). Globally, 2.7 billion people were exposed to filariases (World Health 2006). Susceptible people with severe chronic disabilities generate significant health costs both on the infected persons or on their communities (Wasmuth *et al.* 2008). Consequently, the WHO has sponsored vector and parasite control for these diseases over several decades.

2.2.2.2 *Lymphatic Filariasis*

Lymphatic filariasis (LF) is caused by *Wuchereria bancrofti, Brugia timori* or *B. malayi*. The main cause of LF in Africa, Latin America and the Caribbean is *Wuchereria bancrofti*, while *Brugia* spp. are common in Southeast Asia, Timor and surrounding islands of the Savu Sea (Udall 2007). The parasites reside in the lymphatic vessels and obstruct lymph flow. The main chronic clinical signs are swelling of the genitalia, legs, arms and breasts (WHO 2013). A review of the historical, epidemiological and control

features of LF suggested that the disease existed 1500 BC in Egypt, but the first report of the Mf was in 1863 from hydrocele fluid collected from a Cuban in Paris (Otsuji 2011). The disease is transmitted by mosquitoes and is endemic in 73 Africa, South East Asian and Latin American countries including the Carribean Islands and across the Pacific. Unlike with onchocerciasis, 65% of LF infections are found in South East Asia (WHO 2013). Lymphatic filariasis, also known as elephantiasis, is the second leading cause of chronic disability worldwide (WHO 2012). According to the latest Global Burden of Disease study, about 38.5 million people are infected worldwide (Vos *et al.* 2016). A third of them develop elephantiasis (severe enlargement of the legs and feet), which is the most stigmatizing form of the disease (WHO 2012).

Most patients of LF are usually infected when they were children, but clinical manifestations start appearing in adulthood. Acute forms of LF occur when the skin, lymph nodes and the lymphatics vessels are involved. About 20% of infected people have occult LF (amicrofilaraemia) and 40% of them have kidney damage or manifest urogenital swelling (WHO 2012).

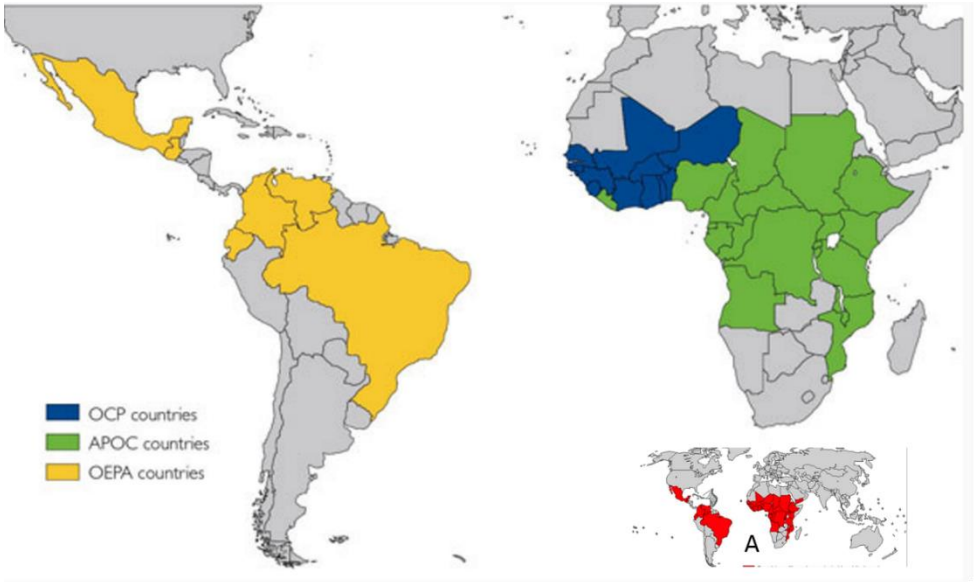
2.2.2.3 *Loiasis*

Loiasis is found only in Africa (Boussinesq 2006). It is caused by *L. loa* (African eye worm) and is transmitted by *Chrysops* flies (*C. silica* and *C. dimidiata*) belonging to the Tabanidae with 13 million people infected. The disease is prevalent among inhabitants of the equatorial rainforest of West and Central Africa including Sudan, Uganda, Zambia, Angola and six other countries and about 14.4 million people live in high-risk areas (Zouré *et al.* 2011). The prevalence of loiasis varies from one region to the other and could be as high as 54% (Boussinesq and Gardon 1997; Hassan *et al.*

2011). Adult *L. loa* reside in fatty subcutaneous tissues and migrate subcutaneously, causing local “Calabar” swellings. The parasite can be seen when the eye is affected, as it migrates across the eye subcutis (Eveland *et al.* 1975). Depending on geographical regions where infection occurs, the disease is known as subcutaneous filariasis, Calabar swelling, Fugitive swelling, Tropical swelling and African eye worm. The diagnosis of loiasis is difficult because most often the condition is asymptomatic and amicrofilaremic (Eveland *et al.* 1975). Acute symptoms of the disease such as lymphedema, episodic angioedema accompanied by localized skin eruptions and pruritus usually last only for 1-3 days (Eveland *et al.* 1975; Boussinesq *et al.* 1998). During the chronic phase, there are cyst-like enlargements of the connective tissue around the sheaths of damaged muscle tendons. These swelling are very painful when touched.

Loiasis is also a common infection of non-human primates. However, they are transmitted by *Chrysops langi,* which is different from the human vector, and there is no cross-infection between human and simian loiasis (Duke 1957). IVM causes rapid death of Mf and can evoke very severe adverse effects (SAEs) such as skin rashes, encephalopathy and finally death (Haselow *et al.* 2003). Because of these SAE, the use of IVM during control of onchocerciasis is not recommended in areas of co-infection with loiasis (Boussinesq *et al.* 1998). Consequently, an epidemiological map of the distribution of loiasis in onchocerciasis infested zones was imperative, and a rapid assessment procedure “RAPLOA” was established to facilitate the mass distribution of ivermectin (Kelly-Hope *et al.* 2012). Ivermectin is not administered in areas of high onchocerciasis endemicity (Thomson *et al.* 2004; Baker *et al.* 2010;

Zouré *et al.* 2011) that also have a high risk of loiasis (Kelly-Hope *et al.* 2012; Wanji *et al.* 2012).



Source: WHO (A) and USAID- http://www.neglecteddiseases.gov/target_diseases/onchocerciasis/

Figure 2.2: Distribution of onchocerciasis control programmes worldwide (OCP, Onchocerciasis Control Programme; APOC, African Programme for Onchocerciasis Control; OEPA, Onchocerciasis Elimination Program for the Americas). The insert (A) marked in red has Yemen added to the OCP, APOC and OEPA countries.

2.2.2.4 *Onchocerciasis*

Onchocerciasis is commonly known as river blindness. However, blindness is one of the irreversible clinical manifestations that begins with visual impairment and skin depigmentation or deformation caused by Mf that migrates out of gravid *O. volvulus*. The disease is prevalent in 31 African countries, part of Latin America and Yemen (Figure 2.2 A).

2.2.2.4.1 Epidemiology

Onchocerciasis is a riverine disease affecting populations that carry out economic activities along unprotected streams, lakes, estuaries or rivers. It is endemic in 31 tropical African countries (Figure 2.2). In Latin America, transmission has been stopped in all but one focus (WER 2012; Convit *et al.* 2013; Rodriguez-Perez *et al.* 2013). However, western Yemen on the Arabian Peninsula remains endemic, with an estimated 350,000 – 400,000 people at risk (Al-Kubati *et al.* 2018). Onchocerciasis affects 15.5 million people (Vos *et al.* 2016), while 125 million people are exposed to the disease worldwide. Ninety-nine percent of the infected people are poverty-stricken Sub-Sahelian Africans (Hall and Pearlman 1999; MD 2001; APOC/WHO 2011).

To facilitate the implementation of control measures geared towards eradication of the disease by 2025, African countries endemic for onchocerciasis were mapped by using "Rapid Epidemiological Mapping of Onchocerciasis (REMO)" taking into consideration the presence of vector flies (*Simulium* spp.) and their breeding sites (Ngoumou *et al.* 1994; Macé *et al.* 1997). Formerly, only 10 West African countries were part of the Onchocerciasis Control Programme (OCP) for West Africa (Davies 1994; Macé *et al.* 1997; Takougang *et al.* 2002). After REMO, 20 more countries in Africa were found to be endemic (Figure 2.3) and included in the African Programme for Onchocerciasis Control (APOC) countries. In Cameroon for example, half of the population are at risk (Macé *et al.* 1997), about 5 million suffered from onchocerciasis and there were 30,000 confirmed cases of blindness (CAS 2012). The REMO of Cameroon (Figure 2.4) illustrates onchocerciasis-endemics areas in Cameroon targeted for community-directed treatment with ivermectin (CDTI).

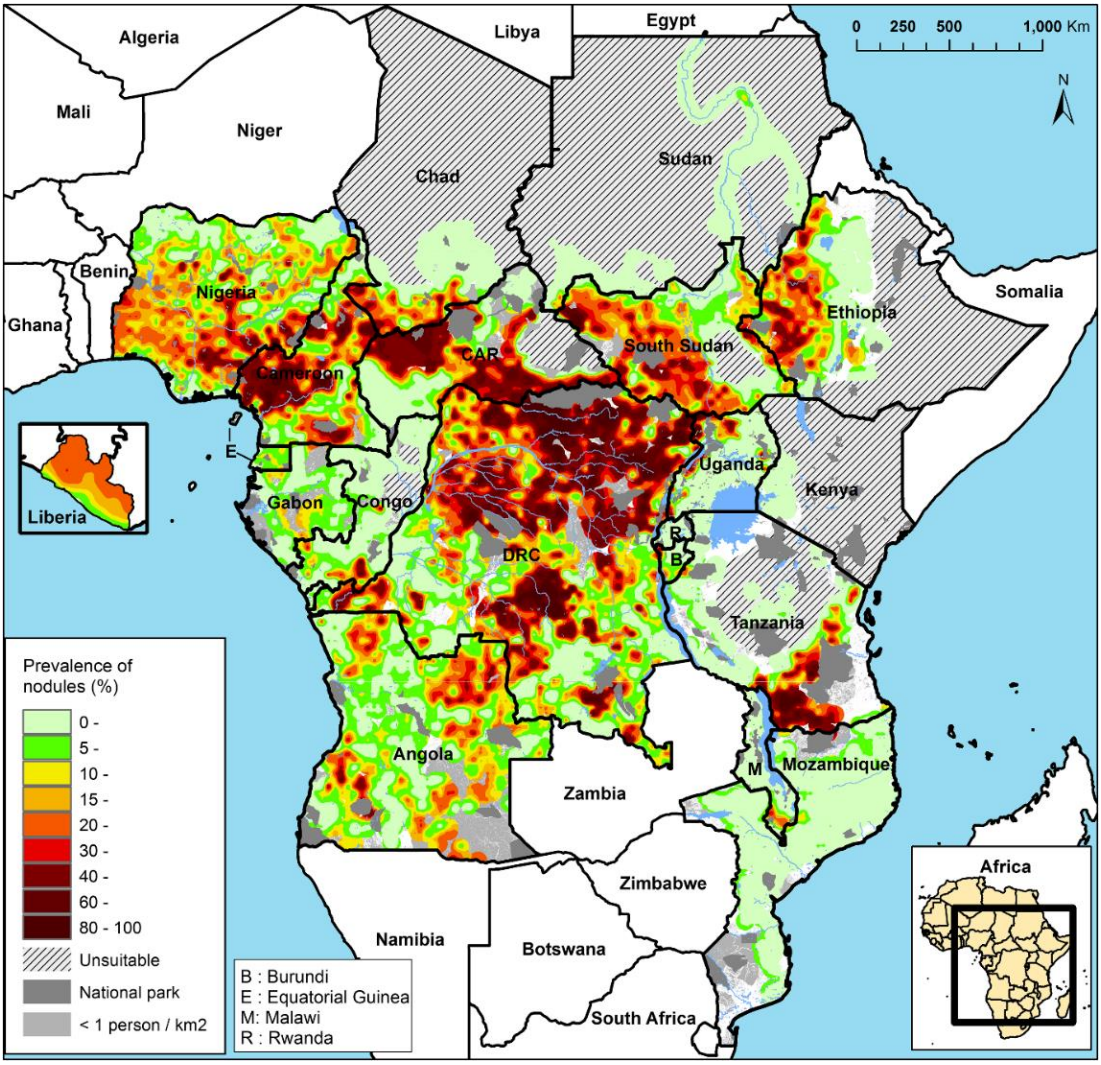


Figure 2.3: "REMO" onchocerciasis endemicity in the additional 20 African countries placed under the African Programme for Onchocerciasis Control. Source of map: (Zoure *et al.* 2014)

Unlike most infectious diseases, *O. volvulus* naturally infects and causes disease only in man. In chimpanzees, experimental infections lead to transient patency (Soboslay *et al.* 1992; Lüder *et al.* 1993; Eberhard *et al.* 1995). The closest biological relative of *O. volvulus* is *O. ochengi (Morales-Hojas et al. 2006),* which has been very useful in the study of the biology, immunology and pharmacology of onchocerciasis (Graham *et al.* 2000; Trees *et al.* 2000). The female worms of both *O. ochengi* and *O. volvulus*

are immobilised within nodules (Figure 2.5). In most parts of Africa (95%), onchocerciasis is transmitted by *Simulium damnosum sensu lato (sl)*, which also transmits *O. ochengi*. However, *S. neavei* is the principal vector in Ethiopia, Uganda, Tanzania and the Democratic Republic of the (Fischer *et al.* 1993) and *S. metallicum sl, S. ochraceum,* and *S. exiguum* in the Americas (Basáñez *et al.* 2002).

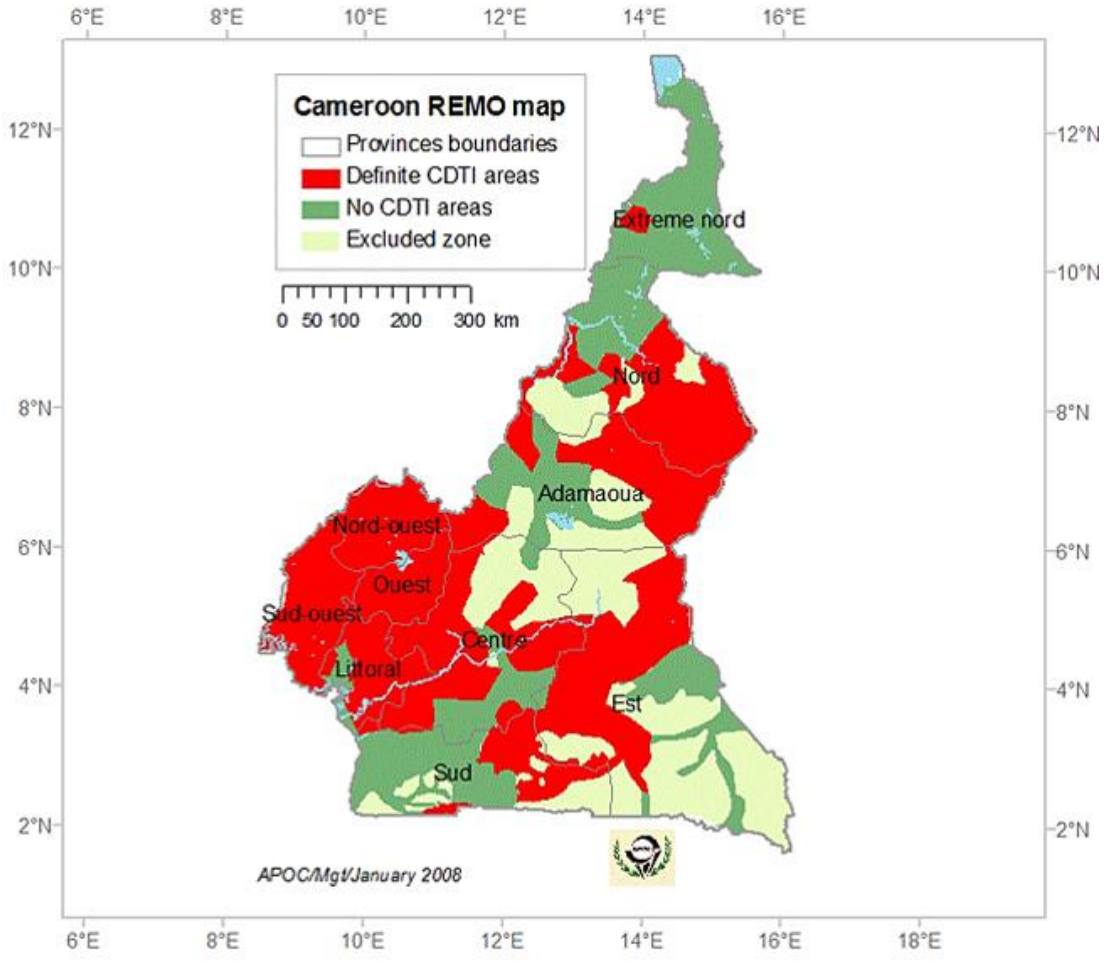


Figure 2.4: Map of Cameroon showing Onchocerciasis endemic areas targeted for Community Directed Treatment with Ivermectin (CDTI). Source of map: WHO http://www.who.int/apoc/countries/cmr/en/.

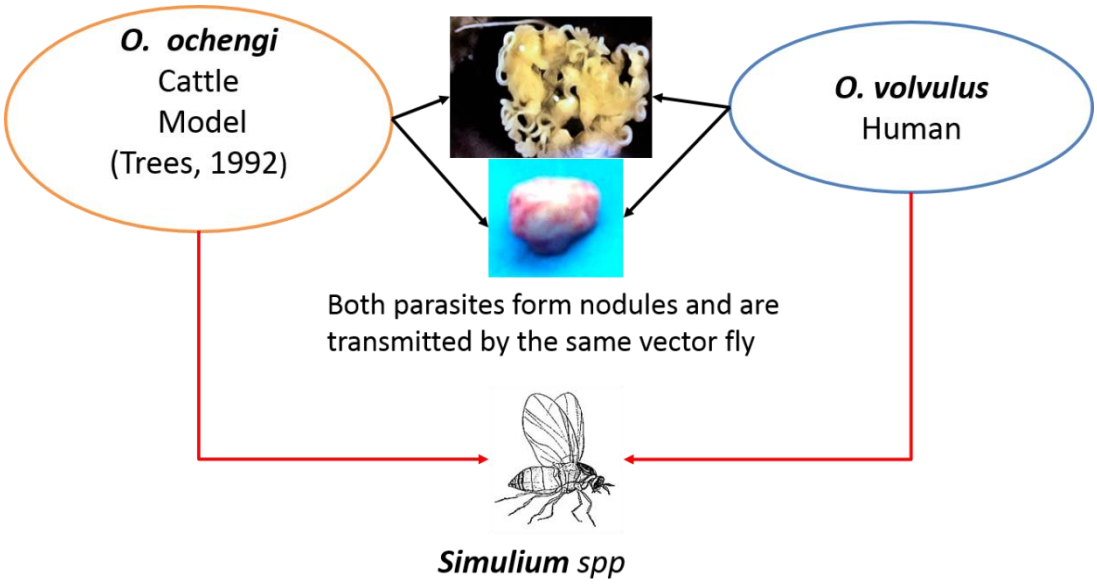


Figure 2.5: Practical reasons for use of *O. ochengi* as an optimal research model for *O. volvulus.*

Figure 2.6: Forms and clinical manifestation of onchocerciasis. Acute papular onchodermatitis (APOD) is characterised by reddening of the skin. The other manifestations, from chronic papular onchodermatitis (CPOD) and lichenified onchodermatitis (OD) to visual impairment, which often leads in complete blindness, are chronic forms.

2.2.2.4.2 Clinical Manifestations

The earliest signs of infection of *O. volvulus* in humans are fever, joint pains, pruritus, swollen glands and associated skin pathologies (Blacklock 1927) preceding visual impairment (Buttner *et al.* 1982; Albiez *et al.* 1988; Petit *et al.* 1992; Wanji *et al.* 2002; Brattig 2004). The pathognomonic sign of onchocerciasis (Figure 2.6) is the presence

of palpable subcutaneous nodules or worm “tumours” called onchocercomata. The subcutaneous nodules can be easily palpated as small, soft and smooth, loose subcutaneous tumours or large, hard irregular aggregates of nodules on the head, arm and ribs, or associated with the elbow and knee joints (Blacklock 1927). In cattle, the nodules of *O. ochengi* are intradermal and are identified as smooth, soft spherical dermal tumours situated mostly between the hind limbs, udder or scrotal sac and ventral abdomen (Wahl *et al.* 1994). However, nodules can be formed on any part of the body.

Chronic symptoms of onchocerciasis are consequences of immunopathology over a long period of time (Nanduri and Kazura 1989; Udall 2007). Consequently, visual impairment and blindness are high amongst adults. Before the introduction of the onchocerciasis control programmes, one- third of patients infected with *O. volvulus* became blind (Prost 1986), and mortality was high due to social stigmatisation as much as the morbidity of the disease (Kirkwood *et al.* 1983; Ovuga *et al.* 1995; Boatin and Richards Jr 2006). Retrospective studies conducted by Little *et al.* (2004) between 1971 and 2001, showed that 5.2% of the world’s onchocerciasis-related mortalities were due to blindness.

Although onchocerciasis is caused by a single infective agent, clinical manifestations vary from location to location and depend on the immunological response to infection. A chronic hyper-reactive form of onchodermatitis emanating from strong cellular and humoral immune responses generated against Mf (Buttner *et al.* 1982; Brattig *et al.* 1987), and characterised by very low Mf densities, few onchocercomata

and relatively lower serum triglycerides, is “sowda” (Mpagi *et al.* 2000). Sowda, also known as reactive onchodermatitis (Hoerauf *et al.* 2002; Magdi M. M. Ali *et al.* 2007), was first described in Yemen and Sudan, but the condition occurs in less than 1% of patients in other endemic regions. Pathologies are limited most often to a single lower extremity, though both limbs may be involved (Figure 2.6). Older patients in the hyper-endemic areas may present sowda (Stingl *et al.* 1984), characterised by pretibial depigmentation portraying a “leopard skin” appearance (Bradley and Unnasch 1996).

Epilepsy is a neglected symptom of onchocerciasis that has only recently been recognised (Colebunders *et al.* 2017). Consequently, this clinical manifestation is not often included in the calculation of the disease burden. Studies have shown that for every 10% increase in onchocerciasis, there is a corresponding increase of 0.4% in cases of epilepsy across all the *Onchocerca*-endemic African countries (Pion *et al.* 2009). Controlled experiments in Cameroon and Central Africa Republic showed that the Mf level of epileptic patients in oncho-endemic regions is higher but not statistically significantly different from those of the control population (Boussinesq *et al.* 2002). Though there is a high probability that epilepsy may be associated with high Mf levels in *Onchocerca* patients (Kaiser *et al.* 2011) more studies will need to be conducted with larger sample sizes to resolve the relationship between epilepsy and onchocerciasis. Such studies are becoming increasingly difficult to execute given the success recorded by the ongoing onchocerciasis control programmes in reducing skin Mf load.

2.2.2.4.3 Life Cycles of *Onchocerca spp*

The life cycle of both *O. volvulus* and *O. ochengi* are like those of other filarial infections described earlier under reproduction (section 2.1.4) and summarised in Figure 2.7. Ingested Mf penetrate the gut walls of the vector fly into the hemocoel through the digestive cells and the basal lamina (Bain and Babayan 2003). In 3 to 5 days, the L1 undergoes two obligatory moults while migrating to the head to become infective larvae or L3 (Blacklock 1927). In the process, the vector's tissues are damaged and in very heavy infections, the vector dies within 10 days (Bain *et al.* 1976; Chabaud *et al.* 1986). The duration of blood feeding during which L3 are transmitted to humans or cattle is about 5 minutes (Nanduri and Kazura 1989).

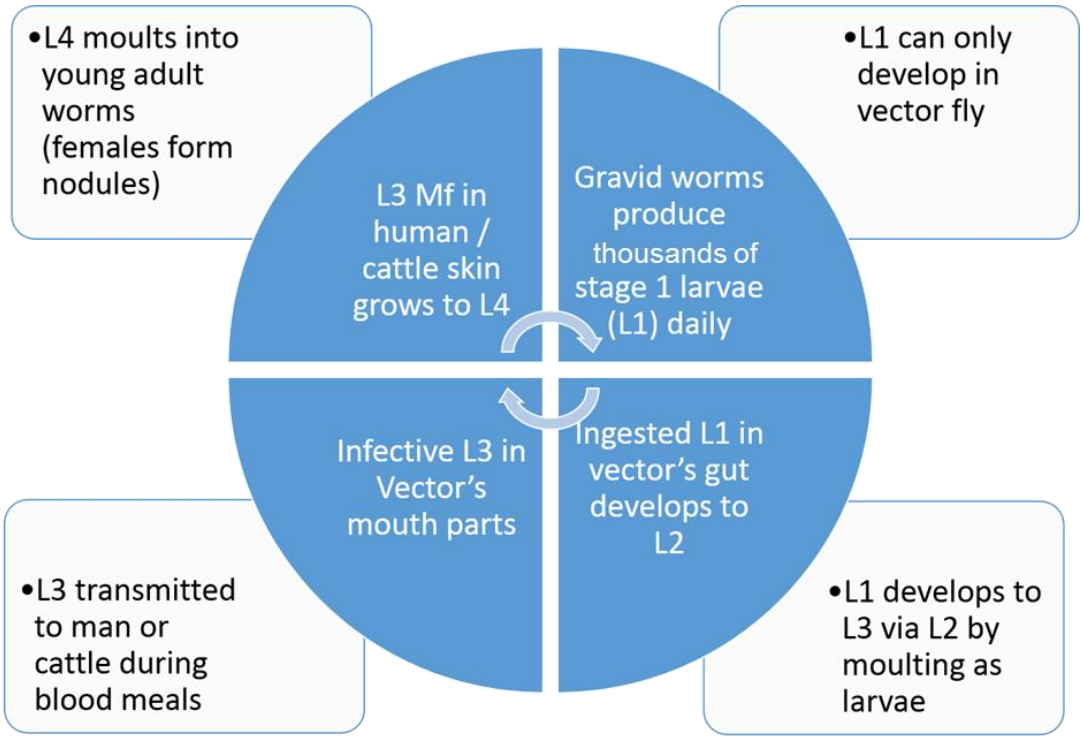


Figure 2.7: Life cycle of onchocerciasis

The vector fly, *S. damnosum,* usually flies at very low altitudes (about 50 cm from the ground or water surface). Therefore, areas such as the ankles, hands, and head (in children) in humans are most susceptible to bites and infection (Wahl *et al.* 1994). While standing in a stream to drink, the abdomen, udder or testicles of cattle are closer to the surface of the waters in which the fly breeds and are the most frequently bitten sites for transmission of infection. The chances of having an infective bite are usually higher in the evening than the early mornings, and the L3 present in the saliva is deposited to facilitate entry into the skin after every blood meal (Bain and Babayan 2003).

An infective L3 is 586-760 µm long and very motile. It will migrate into the subcutaneous tissues' superficial lymph vessels where it grows and moults into fourth stage larvae (L4) within 3 months (Wahl *et al.* 1994; Achukwi *et al.* 2000). The L4 then moults into a young adult and matures within 18 – 34 months (Chabaud *et al.* 1986; Bain and Babayan 2003). A fertile female may live for 10 to 15 years while maintaining good fertility and producing between 1,300 to 1,900 L1 (0.3 mm long) per day (Burnham 1998).

2.2.2.4.4 Diagnoses of Onchocerciasis

The diagnosis of onchocerciasis is based on the history of the origin of patient (REMO), clinical manifestations, laboratory tests and the isolation and identification of worms or microfilariae.

2.2.2.4.4.1 Clinical and Epidemiological Diagnosis of Onchocerciasis

The first rapid epidemiological diagnostic tool for onchocerciasis is REMO (Figure 2.3), a map that demarcates the risk zones in Africa. The first clinical symptom of early infection is acute papular onchodermatitis (APOD) and subsequently the chronic papular onchodermatitis (Figure 2.6). Before the appearance of clinical signs, subcutaneous *Onchocerca* nodules can be palpated. A clinical test kit has been developed to provoke a skin manifestation (the Mazzotti test) using diethylcarbamazine (DEC)-soaked gauze. The anthelminthic is applied on both the diseased and healthy areas of skin for 1 or 2 days (Stingl *et al.* 1984), and the appearance of more than 20 fresh papules on the infected skin alone is indicative of a positive Mazzotti reaction. This technique does not detect pre-patent infections, is time consuming and not patient-friendly (Stingl *et al.* 1984).

2.2.2.4.4.2 Laboratory Diagnosis

The most accurate laboratory technique used to diagnose onchocerciasis is the skin snip test for active disease or isolation of adult worms from nodules. Skin snips or biopsies are taken from specific sites, cultured in any physiological solution at 37^{o}C and examined for the presence of Mf. This gold standard technique is less sensitive to low Mf load and is not recommended for the monitoring of the effectiveness of post-ivermectin therapy (Boatin and Richards Jr 2006). The Roswell Park Memorial Institute (RPMI) medium 1640 is the media of choice to monitor drug efficacy on parasites. Optimum results are obtained when antibiotics are added to prevent bacterial overgrowth and the skin is digested with collagenase for maximal release of Mf.

Immunoassays for anti-*Onchocerca* antibodies, especially targeting the Ov16 antigen, have been available for many years in both ELISA and rapid card formats (Weil *et al.* 2000). This is becoming more widely used in post-control surveillance as the "SD BIOLINE Onchocerciasis IgG4 (Ov16) rapid test" following increased accessibility via the non-profit organisation PATH (Dieye *et al.* 2017). However, the Ov16 test is only appropriate for use in monitoring children (less than 11 years of age) as "sentinels" of transmission during post-elimination surveillance, as adults can remain seropositive for many years after transmission has stopped, due to either persisting infection or maintenance of antibodies after worm clearance (Golden *et al.* 2016). Sensitive diagnostic tools for active infection, including PCRs (Lloyd *et al.* 2015) and isothermal amplification methods (Lagatie *et al.* 2016) for use on skin snips, have been developed but are not yet widely used. Identification on non-invasive biomarkers of adult worm infections is a major research priority for onchocerciasis (Vlaminck *et al.* 2015).

2.2.2.4.4.3 Pathogenesis of Onchocerciasis

The path taken for any disease to establish is important in designing an effective disease control strategy. Immediately after deposition of L3 into the skin, the parasites quickly penetrate the lymph vessels to evade immune attack, establish and develop into adult worms by forming a nodule around the female. It is unclear how L3s migrate through tissues into the lymph vessels. The presence of cephalic hooks at the anterior end of the Mf suggest an important role in locomotion and invasion of tissues (Bain and Babayan 2003). The pathogenesis of nodule formation is also unclear and will remain so until time when *in vitro* studies can be performed on the

development of *Onchocerca* species. During the growth phase of the worm and establishment of the nodule, there seems to be limited pathology caused to the host as the parasite is sequestrated and immunity modulated to favour establishment. The clinical manifestations of infections from endemic and hypoendemic areas differ. In endemic areas, there is immune hyporesponsiveness, heavy nodule loads and skin Mf. There is widespread distribution of mild dermatitis and depigmentation (Soboslay *et al.* 1997), with limited involvement of the lymph nodes. At maturity, fertile females release hundreds of Mf into the vagina from where they migrate into nodule fluid and are carried along in the fine lymphatics vessels of the skin, from where they accidentally escape out into the subcutis to elicit allergic immune responses that cause pruritus, inflammation and reddening of the skin. Release of endobacteria *Wolbachia* from dead Mf further exacerbates the pathology (Boatin and Richards Jr 2006). In hypoendemic regions, hyperresponsiveness is observed, resulting in lower worm establishment and low or amicrofilaridermia (Henson *et al.* 1979; Brattig *et al.* 1994), but greater morbidity is caused by immunopathology (Kirkwood *et al.* 1983).

Most of the circulating Mf die and are carried by lymph flow to the lymph nodes where they are phagocytized by immune cells. Daily, lymph nodes draining affected regions are over worked become hypertrophic and the lymphatic system fails. Hydrocele of the inguinal region ensues. With time, the failed lymph node atrophies and collapses to hang in the inguinal pouch created by the oedematous skin.

2.2.2.4.5 Treatment and Control of Onchocerciasis

2.2.2.4.5.1 Treatment

There is no recommended treatment against onchocerciasis that is macrofilaricidal. Antibiotics of the tetracycline family can kill adult worms, but their use is restricted to individual cases. Melarsomine dehydrochloride is an organoarsenical macrofilaricide used to treat heartworm in dogs (McTier *et al.* 1994; Bowman and Atkins 2009), but cannot be used in humans because of toxicity. It is used for research purposes in cattle as a positive control for macrofilaricidal activity, but the drug must be administered at a very high dose. Suramin a trypanoside used both on animals and humans (Nok 2003) had some pathological effects on embryos but was not macrofilaricidal (Renz *et al.* 1995).

Ivermectin is the lone drug available for use in the mass control of human onchocerciasis. It is microfilaricidal and has limited effects on adult worm viability. Although nodules can be surgically removed (Burnham 1998), circulating Mf with a lifespan of six months to two years would have to be treated with ivermectin. IVM was discovered in the 1970s as a veterinary parasiticide and its use in the human pharmacopeia was validated in the mid-1980s (Campbell W *et al.* 1984).

The first anthelminthic used to control onchocerciasis in 1949 by Hawking and Laurie was diethylcarbamazine-citrate (DEC). It kills only Mf and not the adult worms (Adams and Woodruff 1953). Moreover, DEC was contraindicated in areas with high endemicity because of severe side effects, especially affecting the eye (Oomen 1969; Fuglsang and Anderson 1973; Fuglsang and Anderson 1974). DEC is less effective in

controlling disease in patients with low Mf load. Steroidal anti-inflammatory drugs could be administered to reduce the side effects of DEC (Awadzi and Gilles 1992).

2.2.2.4.5.2 Onchocerciasis Control Programme for West Africa

The WHO launched the Onchocerciasis Control Programme in West Africa (OCP) in December 1974 (Roberts *et al.* 1967; Waddy 1969) in collaboration with the United Nations Development Programme (UNDP), the International Monetary Fund (IMF) and the World Bank to interrupt disease transmission via vector control (Senghor and Samba 1988; WHO 2018). Weekly aerial applications of larvicides on *Simulium* breeding sites were conducted in 11 West African Countries (Figure 2.4). To eradicate onchocerciasis, the programme was to be applied for at least 14-16 years; equivalent to the lifespan of an adult female worm. All measures were taken to avoid the vector developing insecticide resistance (Hougard *et al.* 1997) and environmental degradation (Leveque *et al.* 1988; Calamari *et al.* 1998; Crosa *et al.* 1998). The main constraints to the programme were cost and accessibility. The program reclaimed 1,200,000 km^2 of land and protected 30 million people from 11 OCP countries (Hougard *et al.* 1997; WHO 2018), thereby greatly reducing onchocerciasis as a public health and socio-economic problem in these areas (Senghor and Samba 1988; De Sole and Remme 1991). About 25 million hectares of abandoned agricultural land were reclaimed, allowing 18 million births in areas freed of risk of getting blind from onchocerciasis (De Sole and Remme 1991; Molyneux 1995; Boatin *et al.* 1997). As more than 100 million people were still living in areas where transmission took place outside the OCP boundaries (Davies 1994; María-Gloria Basáñez *et al.* 2006), the

African Programme for Onchocerciasis Control (APOC) was created in 1995 (Taylor *et al.* 2009).

2.2.2.4.5.3 The African Programme for Onchocerciasis Control

Following the discovery of IVM (Campbell W *et al.* 1984) and confirmation of its superior clinical efficiency against *O. volvulus* compared to DEC in 1980 (Greene *et al.* 1985; Lariviere *et al.* 1985; Diallo *et al.* 1986), the manufacturers of Mectizan® (Merck, Sharp and Dohme) in 1987 provided free drugs to be used in the control of onchocerciasis for as long as it would take to eradicate the disease (Colatrella 2008). This gave birth to APOC in 1995, and the OCP mandate extended to 2002. Twenty additional *Onchocerca* exposed countries in Africa (Figure 2.3) were added and mass drug administration of IVM tablets (Mectizan®) at a frequency of once per year was instituted (Delisle *et al.* 2005). In the Americas, the Onchocerciasis Eradication Programme for the Americas (OEPA) was initiated in 1992. Hyperendemic regions were earmarked for eradication of the disease. Thirteen regions had foci of onchocerciasis in six Latin American countries (Sauerbrey 2008). An annual or biannual Mectizan® distribution (the latter used predominantly by OEPA) and administration was adopted, aimed at eradicating onchocerciasis (in OEPA) or controlling it as a major public health and socio-economic developmental problem (in APOC). Given the success achieved such as apparent abrogation of transmission in certain regions of West Africa using IVM alone (Diawara *et al.* 2009; Traore *et al.* 2012), WHO broadened the remit of APOC to include elimination of onchocerciasis in 23 of 31 African countries by 2020 (Diawara *et al.* 2009; Traore *et al.* 2012). In 2015, APOC officially closed and onchocerciasis control is now devolved to the governments

of endemic countries under the umbrella of “ESPEN” (Expanded Special Project for Elimination of Neglected Tropical Diseases), potentially diluting efforts to break transmission across the continent (Hopkins 2016).

However, the use of macrofilaricidal drugs would have a higher potential for the elimination of onchocerciasis (Wanji *et al.* 2009). The use of doxycycline in a 6-week course of community-directed intervention (CDI) has been cost effective (Turner *et al.* 2010) and is now being implemented in loiasis-endemic areas of Cameroon. Doxycycline has been recommended as an individual treatment for some years (Taylor *et al.* 2010), and is now known to be as effective after a 4-week course compared with the original 6-week course (Walker *et al.* 2015).

2.2.2.4.6 Mechanism of Action of Drugs Used

2.2.2.4.6.1 Ivermectin

IVM is administered at a dosage of 150 µg/kg. A single dose is effective against the Mf for 3 – 6 months (Newland *et al.* 1988; Alexander *et al.* 1993) and affects the fertility of adult worms, sometimes stopping disease transmission following an annual treatment regimen in areas with seasonal transmission when administered for >15 years (Plaisier *et al.* 1995; Diawara *et al.* 2009). IVM selectively binds to parasite glutamate-gated chloride channels and has high affinity to nerve or muscle cells of invertebrates, causing either hyperpolarization (Bennett *et al.* 1988) or depolarization (Pemberton *et al.* 2001). Membrane permeability to chloride ions is increased, and paralysis may cause direct death of the parasitic worms, starvation that indirectly leads to death, or cause the parasite to be carried by lymph to lymph nodes for destruction. Glutamate-gated chloride channels are absent in some

mammals, and avermectins have low affinity for mammalian ligand-gated chloride channels. Also, avermectins do not readily cross the blood-brain barrier and their antiparasitic activity targets nerves supplied by the general circulation. IVM penetrates onchocercomata and maintains a high concentration for 5 to 7 days (Cross *et al.* 1997). Adult female worm death reported in Cameroon after a three-monthly regimen of IVM (Duke 2005) might have been due to natural death. IVM also interferes with fertility, causing fatal pleomorphic ovarian neoplasms (Duke *et al.* 2002; Duke 2005).

Evidence of resistance to IVM in humans (Taylor *et al.* 2009; Osei-Atweneboana *et al.* 2011) as well as in animals (Prichard and Prichard 2007) was originally thought to be linked to ATP-binding cassette (ABC) transporters (*e.g.*, P-glycoproteins) and β-tubulin (Bourguinat *et al.* 2007; Prichard 2007). New research using genome-wide sequencing of genetic polymorphisms between "good responder" and "suboptimal responder" worms has revealed that reduced susceptibility to IVM is a complex polygenic trait (Doyle *et al.* 2017). However, another macrocyclic lactone used in veterinary medicine, moxidectin, which is a 16-member pentacyclic lactone belonging to the milbemycin class of endectocides, might be used in humans in future (Cotreau *et al.* 2003; Opoku *et al.* 2018). The major limitation in using IVM in mass drug distributions are in areas coinfected with *L. loa*, but antibiotic therapy with doxycycline could be used in these locations (Wanji *et al.* 2009; Turner *et al.* 2010).

2.2.2.4.6.2 Antibiotics

Antibiotics of the tetracycline family (*e.g.*, oxytetracycline, doxycycline and minocycline) when administered over a long period of time are effective against

filarial worms that harbour the endosymbiotic alpha-proteobacterium, *Wolbachia* (Genchi *et al.* 1998; Bandi *et al.* 1999; Hoerauf *et al.* 1999; A. Hoerauf *et al.* 2000b; Langworthy *et al.* 2000; Rao *et al.* 2002). The effect is indirect. When administered over several weeks (continuous) or months (intermittent), a long-acting formulation of oxytetracycline (at a dose rate of 20 mg/kg body weight once monthly for 6 months) in cattle (Langworthy *et al.* 2000; Gilbert *et al.* 2005), and doxycycline (at 100 or 200 mg per day over a 4-6 week period) in humans (A. Hoerauf *et al.* 2000a; Taylor and Hoerauf 2001; Hoerauf *et al.* 2008b; Hoerauf *et al.* 2009; Walker *et al.* 2015) can be macrofilaricidal and causes the disappearance of *Onchocerca* nodules over a period of 1 to 3 years. Community mass treatment with doxycycline is feasible and has been strongly recommended in loiasis-onchocerciasis coinfected areas (Wanji *et al.* 2009; Turner *et al.* 2010), as filarial nematodes of the *Loa* genus do not harbour *Wolbachia* and are not killed by antibiotics. The macrofilaricidal effect of antibiotics is slow and depends on the sustained depletion of *Wolbachia* on which the filarial nematodes rely for survival in both *in vivo* and *in vitro* studies (Simoncini *et al.* 2001; Taylor and Hoerauf 2001; M. Casiraghia *et al.* 2002; Volkmann *et al.* 2003; Dangi *et al.* 2009; Hoerauf *et al.* 2009). An ineffective antibiotic therapy does have a transient effect on *Wolbachia* depletion, which usually repopulates within 24 weeks post treatment (Gilbert et al. 2005).

Efforts to shorten the duration of an effective treatment by testing other antibiotics such as rifampicin or its combination with tetracyclines or other antibiotics yielded limited successes (Townson *et al.* 2000; Volkmann *et al.* 2003; F.O. Richards, Jr. *et al.* 2007; Specht *et al.* 2008; Bah *et al.* 2014). The immediate post-antibiotic therapeutic

effect is the permanent sterilization of the adult filarial female worms (Hoerauf *et al.* 1999; Hoerauf *et al.* 2001; Taylor and Hoerauf 2001; Hoerauf *et al.* 2003). This is an attractive therapeutic outcome, as no microfilariae are released into the skin (Rao and Well 2002) and hence the interruption of parasite transmission via blood-feeding insect vectors (Albers *et al.* 2012). Evidence confirming *Wolbachia* depletion are derived from polymerase chain reaction (PCR) assay showing clearance of *Wolbachia* DNA (Kramer *et al.* 2003; Volkmann *et al.* 2003; Gilbert *et al.* 2005) and reduction or clearance of bacterial specific hsp60 and *Wolbachia* surface protein (WSP) using an immunohistochemical staining technique (A. Hoerauf *et al.* 2000a; Kramer *et al.* 2003). Penicillin, gentamycin, the macrolides (erythromycin and azithromycin), ciprofloxacin and chloramphenicol were shown to be ineffective against *Wolbachia* in rodent models and *in vitro*, and consequently did not display antifilarial activity (Achim Hoerauf *et al.* 2000; Townson *et al.* 2006).

Tetracycline antibiotics generally inhibit protein synthesis in bacteria by preventing the binding of aminoacyl-tRNA to the ribosomal A site, inhibiting translation and cell growth (Tritton 1977; Chopra 1994; Chopra and Roberts 2001; Griffin *et al.* 2010). It is important to note that for the antibiotic to be effective it must penetrate the cells of the host. In Gram negative bacteria, the tetracycline-magnesium complex is absorbed through the outer membrane. The magnesium then dissociates and the released lipophilic tetracycline diffuses through the lipid inner layers of the cells (Dax 1997). Once inside the cells, it complexes again with magnesium until it comes into contact with a ribosome. With onchocerciasis, the antibiotic will need not only to be

in the nodules in therapeutic quantities but also to be absorbed into the worms to have contact with the *Wolbachia*.

Tetracyclines can also have direct effects on filarial moulting. This was demonstrated *in vitro* on *Brugia* larvae using chemically-modified tetracycline analogues that lack antibacterial activity (Hoerauf *et al.* 1999; Rajan 2004).

2.2.2.5 *Endosymbiotic Wolbachia, its Deletion and Effects on Worm death*

2.2.2.5.1.1 Endosymbiotic Wolbachia

Wolbachia are α-proteobacterial, intracellular, *Rickettsia*-like microorganisms (Huigens *et al.* 2004; Casiraghi *et al.* 2005; Serbus *et al.* 2008). They appear as elongated or spherical, double-membraned bodies richly associated with glycogen in the cytoplasm of cells of the reproductive and other infected organs. The size of *Wolbachia* is similar to that of a mitochondrion when observed with an electron microscope, but may vary (Yen and Barr 1971). *Wolbachia* was first discovered in the *Culex pipiens* mosquitoes and named *Wolbachia pipientis* (Hertig and Wolbach 1924). There are currently 17 phylogenetic super-groups (A-Q) of *Wolbachia* found in >50% of terrestrial arthropods (Weinert *et al.* 2015), a few genera of the Onchocercidae (Fenn *et al.* 2006; Ferri *et al.* 2011) and two genera of plant-parasitic nematodes, *Radopholus* (Haegeman *et al.* 2009) and *Pratylenchus* (Brown *et al.* 2016). Most of the *Wolbachia* found in arthropods belong to super-groups A, B and E, while those of nematodes to super-groups C and D (Bandi *et al.* 1998; Casiraghi *et al.* 2005; Comandatore *et al.* 2013; Gerth *et al.* 2014). *Wolbachia* of the super-group F are found in both nematodes and arthropods. They are transmitted vertically by the

female host through the germline. The mature spermatozoa contain minimal cytoplasm and infected males accounts for less than 2% of vertical transmission (Casiraghi *et al.* 2005). In many arthropod species, uninfected females mated with infected males do not produce viable offspring or have reduced fertility; a phenomenon called “cytoplasmic incompatibility” (Yen and Barr 1971; Huigens *et al.* 2004; Serbus *et al.* 2008). This can also occur as bidirectional incompatibility if the *Wolbachia* strains in infected male and female arthropods differ in genomic copy number and mutations in two prophage-associated genes (Bonneau *et al.* 2018). The females are favoured by the *Wolbachia*, and parthenogenesis, male killing or feminisation of embryos are other reproductive effects observed in infected arthropods. In addition, the ability of *Wolbachia* to inflict phenotypic changes in its host is beginning to be exploited to reduce the transmission of some important arthropod-borne (Glaser and Meola 2010; Mousson *et al.* 2010) and protozoan (Moreira *et al.* 2009; Gomes *et al.* 2017) diseases.

Unlike in some arthropods where *Wolbachia* also affect the fertility of its host (Yen and Barr 1971) and could modulate the arthropod’s ability to transmit diseases, in nematodes, they have been considered nutritional or energy-generating mutualists (Foster *et al.* 2005). They develop within the somatic and germline cells of adult worms as an endosymbiont (Landmann *et al.* 2012). This mutualistic association between *Wolbachia* and filarial nematodes became important when antibiotics of the tetracycline family were shown to retard the development of embryos and larvae (Bandi *et al.* 1999) and eliminate onchocerciasis in cattle (Langworthy *et al.* 2000). The basis for this mutualistic relationship may vary from one *Wolbachia* group to

another and may be immunologic and/or metabolic (Foster *et al.* 2005; Nfon *et al.* 2006; Hansen *et al.* 2011; Darby *et al.* 2012). Efforts to trace the origin of the mutualistic relationship of *Wolbachia* and its host from genomic studies are on-going. Major limitations are serious long-branch attraction artefacts (considering distantly related bacteria as close relatives) in the molecular phylogenetic studies (Bordenstein *et al.* 2009).

2.2.2.5.1.2 Role of Wolbachia in Filarial Nematode

The depletion of *Wolbachia* populations following antibiotic therapy causes an immunological switch around parasitic nematodes from neutrophilia to eosinophilia and worm killing (Nfon *et al.* 2006; Hansen *et al.* 2011). The immediate post-*Wolbachia* depletion did not affect the motility and viability of Mf; however, the adult females were sterilized (Hoerauf *et al.* 2003). The viable Mf, L4 and adult germline or embryos showed extensive non-autonomous apoptosis following tetracycline treatment of *B. malayi* (Frederic Landmann *et al.* 2011). Interestingly, apoptosis was absent in the hypodermal cords (Frederic Landmann *et al.* 2011), although some disruptions to the hypodermal cytoskeleton were observed. The effects of antibiotics were more prominent in gravid females than other adults or stages of filarial worms. In the long term, the development of larval and embryonic worm stages are arrested, while sterilised female worms finally die within 1–2 years (Walker *et al.* 2015). In the cattle model of onchocerciasis, eosinophil degranulation on the surface of the worm following *Wolbachia* depletion contributed towards worm killing (Nfon *et al.* 2006; Hansen *et al.* 2011). *Wolbachia* may also have a mitochondria-like function in the

worms (Darby *et al.* 2012), which together with its metabolic and immunologic role, are vital for worm survival.

Wolbachia released into the tissues during the natural death of Mf or drug-mediated killing may initiate a hyper-immune reaction responsible for the pathogenesis of filarial nematode diseases, particularly, the ocular form (André *et al.* 2002; Keiser *et al.* 2002). *Wolbachia* possesses highly immunogenic proteins and produces chemo-attractants for neutrophils (Bazzocchi *et al.* 2007; Tamarozzi *et al.* 2016). In bovine onchocerciasis, the stimulated neutrophils escape from the blood via capillaries and migrate towards the infected worm cuticle to form a type of micro-physical barrier (Makepeace and Tanya 2016). Neutrophils appear to be harmless to intact worms and are prevented by the worm cuticle from phagocytising the endobacteria. In turn, the neutrophils' dominant coat around the worm prevents eosinophils from attaching to the worm surface. Eosinophils are activated to release toxic granules only when attached to the worm's surface after *Wolbachia* depletion (Hansen *et al.* 2011). However, the exact immunological mechanism of worm killing by eosinophils is not known. *Wolbachia* express surface protein (WSP) (Bazzocchi *et al.* 2007) and lipoprotein (*w*BmPAL) (Power *et al.* 2004) that can inhibit neutrophil apoptosis (Tamarozzi *et al.* 2016). These lipoproteins have also been implicated in disease pathogenesis (Turner *et al.* 2009).

In lymphatic filarial worms, *Wolbachia* synthesises haem and riboflavin (Foster *et al.* 2005) and provide a tremendous iron-buffering potential to their hosts (Brownlie *et al.* 2009; Gill *et al.* 2014). However, the *Wolbachia* of *O. ochengi* (*w*Oo) lacks the

ability to synthesise riboflavin (Darby *et al.* 2012), but may serve as a power house for the worm by provisioning ATP.

2.3 Immunology of Onchocerciasis

Onchocerciasis is an immune-mediated disease caused by an exaggerated response to the presence of Mf and/or *Wolbachia* in the skin after establishment of infection. The rational for the review of the immunology of onchocerciasis is to evaluate the possibility of preventing infection, arresting the establishment of infection and finally treating infected animals and prevent reinfection.

2.3.1 Prophylaxis

In areas endemic for onchocerciasis, less than 5% of persons (Kruppa and Burchard 1999; Hoerauf and Brattig 2002) and cattle (Tchakouté *et al.* 2006) are referred to as putative immune (PI) or endemic normal because they are immune to new infection despite exposure to high levels of disease transmission. Zooprophylaxis amongst Fulani nomads living in areas endemic for cattle *O. ochengi* North of Cameroon, and exposed to natural transmission of *O. ochengi* L3 via *S. damnosum* is characterised by protection from *O. volvulus* infection (Wahl *et al.* 1994). Although it is possible that the presence of cattle with larger respiratory volume than man, hence emitting more CO_2, which attract more flies to them than humans (Sutcliffe *et al.* 1994), it was not the main explanation of zooprophylaxis as experimental evidence on calves experimentally vaccinated with irradiated L3 from *O. volvulus* and challenged with *O. ochengi* L3 showed high levels of protection (Achukwi M *et al.* 2006; Tchakouté *et al.* 2006).

2.3.1.1 *Immunology of Putatively Immune Individuals*

Attempts have been made to characterize the sera or immune cells from PI individuals for specific antibodies or cytokine profiles that could be responsible for PI status. *In vitro* studies confirmed that sera from the infected as well as PI individuals could kill L3 and limit the moulting of L3 to L4 during new infection (Berger 2000). Initially, the immunity in PI individuals were thought to be driven by an antigen-specific Th 1-type response (Elson *et al.* 1995), with interferon gamma being the main cytokine produced (Amambua *et al.* 2006). However, it appears that PI individuals express a mixed Th1/Th2 response characterized by a weaker Th1 than Th2 response (Hoerauf and Brattig 2002). The dominant antigen on an infective larvae OvL3.C1, is a histidine-rich and helix-rich protein with a single transmembrane region (Vincent P.K. Titanji *et al.* 2002; Amambua *et al.* 2006). It elicits strong IgG3 and IgG4 responses, which killed the L3 of parasites and blocked pathology respectively (Meyer *et al.* 1994). The PI individuals produce distinct larval and male worm-specific cytokine responses associated with proliferative reactions to *O. volvulus* antigens (Achukwi M *et al.* 2006; Tchakouté *et al.* 2006; Ziewer *et al.* 2012), similar to those of the hyperactive forms of onchocerciasis (Gregory *et al.* 1997; Murray *et al.* 2005; Cho-Ngwa *et al.* 2010; Sam *et al.* 2011; Babayan *et al.* 2012).

Absence of nodules and skin Mf in persons or animals in endemic areas is not diagnostic of PI. Additional tests are needed to differentiate PI from pre-patent or post-patent infections and Sowda. Certain genetic factors (HLA-D allele: haplotype DQA1*0501 and DQB1*0301) tended to lead to the development of PI status in humans (Meyer *et al.* 1994; Turaga *et al.* 2000). There is strong evidence of negative

correlations between Mf load and the immune response, with persons having low Mf load tending to develop the severest form of the disease (Duke and Moore 1968; Brattig *et al.* 1994). It is obvious that irradiated L3s induce strong protective immunity in animal models (Goff *et al.* 2000; Abraham *et al.* 2004), but L3 vaccines for humans will be difficult to licence for ethical reasons.

2.3.1.2 *Immunogenic Onchocerca Molecules (Vaccine candidates)*

The best immunogenic proteins for use in vaccine production are those that will target the invader at the point of entry to the point of establishment of infection, such as attenuated L3s. As generating L3s for large-scale vaccination schemes is challenging and would raise safety concerns for human use, subunit antigens present in all of the stages of the worms have been evaluated for potential as vaccine candidates (Gregory *et al.* 1997; Joseph *et al.* 1998; Gregory *et al.* 2000; Sam *et al.* 2011). To prevent establishment of worm, antigens present in secretory or excretory products of worms and not having host homologs such as the Abundant Larval Transcript (ALT) only expressed in L2 and L3 and the Cysteine Protease Inhibitors (CPI) are potential good vaccine candidates (Babayan *et al.* 2012). Identified recombinant proteins for experimental vaccine production can have their acidic domain mutated before the immunoregulatory genes (ALT and CPI) are genetically engineered (Joseph *et al.* 1998; Babayan *et al.* 2012). Both ALT and CPI are acidic proteins that contribute to parasite invasion and establishment by modulation of host immune attack (Kalyanasundaram and Balumuri 2011; Babayan *et al.* 2012).

Two forms of the secretory/excretory products ALT-1 and ALT-2 are released by the adult and infective larvae respectively of *Brugia malayi* (Bianco *et al.* 1990; Bianco *et al.* 1995; Wu *et al.* 2004), *O. volvulus* (Wu *et al.* 2004) and *Acanthocheilonema* (Pogonka *et al.* 1999). They are produced in the oesophageal glands and secreted only when the parasites are in the mammalian host (Sam *et al.* 2011). ALT-1 and ALT-2 are secreted larval acidic proteins (SLAPs) belonging to the ALT family (Wu *et al.* 2004; Sam *et al.* 2011). The immunogenic property of ALT-2 is highly linked to its 21 amino acid terminal signal sequence. ALT-2 based vaccines induced both humoral and cell mediated immunity characterised by IgG1 and IgG2a, as well as IL4 and interferon gamma production, with or without adjuvant (Murray *et al.* 2005; Sam *et al.* 2011). Adjuvants are usually added to maintain Th1-Th2 balance essential for protection, so having a vaccine that will not need an adjuvant is of interest given that adjuvants may be toxic or provoke production granulomas (Hernández *et al.* 2008).

Cystatins are protein inhibitors of the cysteine proteases found in all metazoan and plant taxa (Watts 2001) and are close relatives of stefins and kininogens in a superfamily of cysteine protease inhibitors (Villadangos and Ploegh 2000). Cystatins play an important role in blocking the activities of cysteine proteases essential in the breakdown of pathogen proteins during antigen presentation (Gregory *et al.* 1997; Manoury *et al.* 2001; Murray *et al.* 2005; Gregory and Maizels 2008; Cho-Ngwa *et al.* 2010). Cysteine proteases are essential for use by APC, which need to generate intracellular peptides bound to products of the major histocompatibility complex (MHC) class II (Gregory and Maizels 2008). CPI-1 and CPI-2 both constitute another subset of nematode cystatins. The structure and variability of cystatins has been

widely reviewed (Bode *et al.* 1988; Murray *et al.* 2005). Of importance to filarial vaccine research is CPI-2, which has acquired an additional function of inhibiting asparaginyl endopeptidase (AEP) and making nematode cystatins unique (Murray *et al.* 2005). Unlike CPI-2 which is present in all the stages of filarial nematodes except Mf, CPI-1 and -3 are expressed in the vector during the late stages of the infective larvae (Gregory *et al.* 1997). They lack the conserved glycine residue in the N-terminal region (Gregory *et al.* 1997) and have only 25% amino acid similarity with CPI-2 in *Brugia malayi* (Anand *et al.* 2011; Sam *et al.* 2011). The CPI-1 cDNA has a 22-nt nematode spliced leader sequence (SL-1) at the 5' end immediately before the start codon, a 19 amino-acid predicted signal sequence, and is abundantly expressed in all L3 EST datasets (Vincent P. K. Titanji *et al.* 2002; Cho-Ngwa *et al.* 2010). The limitation of this subset of cystatins to the infective larvae also makes it a potential candidate for filarial vaccine production.

Another important vaccine candidate identified in secretory products from L3s of most filariae is Vespid Allergen Homologue-like protein (VAH) (Anand *et al.* 2007; Gregory and Maizels 2008; Kalyanasundaram and Balumuri 2011). In *B. malayi*, recombinant proteins or a DNA vaccine encoding BmALT-2 and BmVAH elicit substantial Th2 (IgG1/IgG3) and Th1 (IgG2a) immune responses respectively in jirds and mice (Anand *et al.* 2011).

2.3.1.3 *Vaccination Trials on Onchocerciasis*

The initial approach to filarial vaccine research was to compare sera between the infected and uninfected and monitor sera from L3-vaccinated animals for

identification of antigens (Sam *et al.* 2011; Babayan *et al.* 2012). Then subunit vaccines produced from identified immunogens did not stimulate sufficient protective immunity (Brattig *et al.* 1994). Another set of targets being researched for potential vaccine candidates are immunomodulators present in secretory and excretory products of the parasites. To survive, the parasites have evolved to produce secretions that facilitate invasion of the host and negatively modulate hosts immune attack. These secretary/excretory products have given hope of a possible vaccine against filarial diseases (Magdi M. M. Ali *et al.* 2007).

Melarsomine-treated and irradiated L3-vaccinated cattle were compared in terms of susceptibility to infection, and after 2 years of continuous exposure to intense natural challenge from infected *Simulium*, significantly lower worm burdens were observed in vaccinated animals compared to controls (Tchakouté *et al.* 2006). In the same study, previously cured cattle were susceptible to reinfection. Similar reports of reinfection after treatment were from suramin treated (Renz *et al.* 1995), ivermectin treated (Njongmeta et al., 2004) and antibiotics treated (Nfon et al., 2007) cattle. Based on cross-reactivity and cross-protection between human *O. volvulus* and cattle *O. ochengi* (Wahl *et al.* 1998; Achukwi M *et al.* 2006; Graham *et al.* 2009), a major cattle experiment was conducted on naïve calves to evaluate eight *O. ochengi* recombinant antigens (onchocystatin inclusive) as a combined vaccine in Cameroon (Makepeace *et al.* 2009). Each antigen was administered separately with either Freund's adjuvant or alum. After 22 months post-exposure only 58% of vaccinated cattle were infected compared to 100% in the controls. However, trials using irradiated L3s induced immune protection in other filarial systems are believed to be

eosinophil-dependent (Brattig NW *et al.* 1991; Goff *et al.* 2000; Klion and Nutman 2004).

2.3.1.4 *Innate Immunity*

This study seeks to elucidate the immunochemotherapeutic interaction in onchocerciasis. An in-depth review of the essential helminth immunology and its relationship to acquired immunity is essential. The innate immune system is the first line of defence operated by a combination of physical barriers, phagocytic cells, cytokines and antibacterial peptides (Delves *et al.* 2011). The skin acts as the primary physical barrier to overcome. In filariasis, the vector fly breaks the skin to inject L3 into the subcutis. After invasion, key immune cells align to coordinate reduction of the danger posed by the invader are tissue macrophages, fibroblast, dendritic, endothelial or epithelial cells. These cells, in addition to neutrophils, eosinophils, lymphocytes, monocytes and other specialised immune proteins located in the cardiovascular system have receptors that cause them to respond to the presence of a foreign body to eject or contain it (Chaplin 2010).

2.3.1.4.1 Innate Immune Receptors

Innate immune cells use sensory molecular complexes (receptors) located either on the surface of a cell membrane or within the cytoplasm to communicate between themselves, with other cells and the external environment. These sensory protein complexes are pathogen recognition receptors (PRRs) (Kawai and Akira 2010). PPRs were first discovered in 1989 as cited by L.A.J. O'Neill *et al.* (2013) and are specialised at detecting damage (or pathogen) associated molecular patterns (D(P)AMP) in pathogens, aged cells, cell mediators or signals (Hoving *et al.* 2014). Each receptor

has specialised sub-units that detect different types of DAMPs or PAMPs. The receptors serve as a link between the innate and adaptive immunity.

In innate immunity, PRRs on sentinel cells are germline-encoded receptors with heterogenous functions. They cause the cell to produce substances that activate innate immune mechanisms. Four subgroups of PRRs have been identified based on the type of molecular moieties they interact with (Delves *et al.* 2011). Toll-like receptors (TLRs) are located on the cytoplasmic membrane or in endo-lysosomes. They are the most abundant type (Delves *et al.* 2011), while C-type lectin receptors (CLR) with carbohydrate recognition are crucial in the control of fungal infection domains (Schroder and Tschopp 2010; Hardison and Brown 2012; Rathinam and Fitzgerald 2016). The third group of PRRs are retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs) essential for nuclear reprograming of somatic cells to induce pluripotent stem cells (Sayed *et al.* 2017). The fourth and most important source of pro-inflammation PRRs are called inflammasomes (Schroder and Tschopp 2010). They are intra-cytoplasmic nucleotide binding domain (NBD) or the nucleotide binding and oligomerization domain (NOD), leucine rich-repeat containing protein receptors (NLR) (Muñoz-Wolf and Lavelle 2016)

2.3.1.4.1.1 NLR Receptors (Inflammasomes)

NLRs form multiple cytoplasmic protein complexes called inflammasomes (Schroder and Tschopp 2010; Rathinam and Fitzgerald 2016) that activate proteolytic enzymes of the caspase 1 cascade while under stress or on exposure to intra-cytoplasmic PAMPs (Lamkanfi and Dixit 2014). Before activation to form inflammasones, NLRs are soluble proteins of a diverse family of receptors that reside within the cytoplasm in

an auto-inhibited state (Delves *et al.* 2011). The N-terminal protein-to-protein interaction motifs recruit proteases or kinases upon activation (Kufer and Sansonetti 2011; Lamkanfi and Dixit 2014; Muñoz-Wolf and Lavelle 2016; Rathinam and Fitzgerald 2016). There is also a central oligomerization domain and C-terminal leucine rich repeats (LRRs) that act as a sensor for pathogen products and through which the NLRs are activated into an inflammasome capable of recruiting either an NF_kB-activating kinase, protease of the caspase family, and IL-1β precursors (Muñoz-Wolf and Lavelle 2016). In addition to playing important roles in the regulation of adaptive immunity, tissue homeostasis and apoptosis, NLR inflammasome complexes may also have roles beyond immune regulation (Kufer and Sansonetti 2011).

2.3.1.4.1.2 Toll-like Receptors

All 10 human TLRs (except TLR-3) are expressed by human neutrophils but fewer TLRs (TLR-1, 3, 4, 7, 9 and 10) are expressed in human eosinophils (Hayashi *et al.* 2003). Eosinophils express a greater quantity of TLR-7, which regulates the expression of adhesion molecules CD11b and L-selectin, than do neutrophils (Nagase *et al.* 2003). Together with TLR-8, TLR7 prolongs the generation of superoxide in eosinophils. TLRs are conserved proteins expressed in animals from invertebrates (Anderson 2000) through to dendritic cells (Kadowaki *et al.* 2001) and macrophages (Menzies and Ingham 2006) in mammals. In addition to mediating proinflammatory reactions and an adaptive immune response, TLRs regulate $CD4^+$ $CD25^+$ T cells (Treg) to supress host immunity (Liu and Zhao 2007). Bovine homologues of human TLRs 1 – 10 have 80 to 95 percent similarity (Menzies and Ingham 2006), the most abundant being TLR-4 and TLR-7 (Brattig *et al.* 2004; Bazzocchi *et al.* 2007). Many authors have reviewed the

complex roles of TLRs in disease control, autoimmune diseases (Nagase *et al.* 2003; Zheng *et al.* 2005; Kubinak and Round 2012; L.A.J. O'Neill *et al.* 2013), tissue repair and maintenance of haemostasis. However, in filarial infection, *W*SP is recognised by TLR-2/4 (Gillette-Ferguson *et al.* 2007) and the *Wolbachia*-derived diacylated lipoprotein (Diacyl *W*oLP; a synthetic fragment of *w*BmPAL) is recognised by TLR 2/6 (Turner *et al.* 2009). Diacyl WoLP induces neutrophil-mediated corneal inflammation via TLR-2/6 pathways in the murine keratitis model, while TLR-2 mediates CXC chemokine production by corneal bone-marrow derived cells (Gillette-Ferguson *et al.* 2007).

2.3.1.4.1.3 C-type Lectin Receptors

The CLRs are transmembrane PRRs of macrophages and neutrophils that specifically bind to β-glucan and mannan ligands of fungal cells to mediate Th1/Th17 immune responses (Hardison and Brown 2012). There are a number of intracytoplasmic CLRs such as dectin 1/2, mannose receptors (MRs), DC-SIGN and Mincle involved in fungal binding and phagocytosis and the activation of innate immunity (Drummond *et al.* 2011). CLRs are sub-divided into 17 sub-groups based on structure and origin (Hoving *et al.* 2014). Ligation depends on carbohydrate recognition domains (CRDs) or C-type lectin domain (CTLDs) and the effect is coordinated with those of TLRs in achieving immunity against diseases (Everts *et al.* 2009). The role of CLRs in helminth infection is dependent on MR and Th2 immune responses (Harnett *et al.* 2002; van Die and Cummings 2017). The MR has multiple carbohydrate recognition domains with a calcium-independent R type domain that binds glycans with a non-reducing terminal 3-O-sulfated galactose or 3/4-O-sulfated-N-acetylgalactosamines (Gentil *et al.*

2012).The CRD of CLR can also interact with collagenase and may have a role in filarial infection that goes beyond immune modulation.

2.3.1.4.1.4 Rig-1-like Receptors

The RLRs have two homologues: melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Sayed *et al.* 2017). They are cytoplasmic receptors that act as sensors of viral RNA and target PAMPs that escape detection by TLRs or act as a relay signal from TLRs specialised for detection of nucleic acids (Sayed *et al.* 2017). As with other receptors, RLRs have three domains. The RIG-1 and MDA5 have N-terminal regions that contain caspase recruitment and activation domains (CARDS) (Loo and Gale 2011). Though LGP2 lacks CARDS, it functions in MDA5 signalling. The central domain which interacts with viral particles harbours DExD/H box RNA helices. RIG-1 recognises RNA sequences harbouring a triphosphorylated 5'end, while MDA5 is activated by double stranded RNA (polyIC) (Pichlmair *et al.* 2009). The ligands for LGP2 are yet to be discovered. The RLRs so far have no role in helminth infection and neither is there an evidence for their involvement in the control of *Wolbachia* products.

2.3.1.4.2 The Role of Macrophages

Macrophages are phagocytic leukocytes produced by the bone marrow (Delves *et al.* 2011). During inflammation, activated local macrophages (Epelman *et al.* 2014) are reinforced by monocytes that have extravasated into body tissues during proinflammation. However, in helminth infections, resident macrophages are capable of replication via alternative activation and proliferation (Jenkins *et al.* 2011) to produce cytokine and chemokines that stimulates Th2 immune response (Allen *et*

al. 2008). Macrophages are universally present within all body tissues in many functional phenotypes. Two forms of macrophages exist based on their origin: Embryonically-derived tissue macrophages also known as sentinel cells or tissue scavengers and bone marrow derived monocytes transformed into macrophages only after extravasation (Epelman *et al.* 2014). Macrophages regulate Th1, Th2 and other cytokines and chemokines involved in tissue homeostasis. They are equipped with several receptors that detect abnormalities on cells or danger signals from obnoxious agents. In humans, classical activation of macrophages (M1) is stimulated by gamma interferon (IFN_{γ}), lipopolysaccharides (LPS) and granulocyte macrophage colony stimulation factor (GM-CSF), usually stimulated by non-self-proteins from bacterial and viral particles (Epelman *et al.* 2014). Human M1 macrophages express high levels of major histocompatibility complexes (MHC) class II and CD68 markers and secrete large amounts of proinflammatory cytokines such as IL-1_{β}, tumor necrosis factor (TNF), IL-12, IL-18 and IL-23 that activate Th1 and Th17 cells to mediate cytotoxicity and the production of nitrous oxide from L-arginine (Gentil *et al.* 2012). Macrophages routinely monitor nearby cells for normal function and cause infected cells or aged cells to die by apoptosis. M1 macrophages then engulf the apoptotic cells or pathogen by phagocytosis, process non-self-proteins from the digested pathogen, and present them via the MHC I pathway to cytotoxic $CD8^{+}$ T-cells to activate Th-1 driven immune responses and ultimately memory cells to memorize the antigen for a faster response on second exposure (Zhang and Bevan 2011). When M1 macrophages sense danger, they produce Th1 cell attracting chemokines (CXCL 9 and CXCL 10) and release them to direct neutrophils first to the site of infection

(Delves *et al.* 2011). MHC class II and CD68 co-stimulate CD80 and CD86 on M1 macrophages.

The alternative activation mechanism of macrophages is predominant in response to helminth infections. There are four phenotypes of alternatively activated macrophages (M2). The M2a subtype is stimulated by IL-4, IL-13, helminths and yeast infections; M2b by immune complexes (ICs), IL-1R receptor ligand and LPS; and M2c by tumour growth factor beta (TGF_{β}), IL-10 and glucocorticoids (GCs). M2d are distinct from the others and stimulated by IL-6, leucocyte inhibition factor (LIF) and adenosine (Rőszer 2015). The M2a macrophages produce Th2 cell attracting chemokines CCL17, CCL22 and CCL24, while M2b macrophages produce chemokines CCL5, CCL10 and CCL16. The remaining M2c and M2d macrophage phenotypes produce chemokine CCL1 and CCR2, respectively. The cytokines produced by these M2 macrophage subunits vary, but they all produce IL-10. M2a, c and d release TGF_{β} M2b and d produce TNFα. The M2b macrophages secrete IL-1 and IL-6, as for those produced by M1 macrophages. Also, M2d macrophages share in the production of IL-12 with M1 macrophages. M2 macrophages function as immune modulators and promote toleration of infection, tissue remodelling, phagocytosis, tumour formation and control of parasitism (Esser-von Bieren *et al.* 2013). Apart from their origin, there is no clear-cut major functional difference between blood-derived classical $Ly6c^{Hi}$ monocytes and tissue macrophages. The origin and functions of tissue macrophages have been reviewed (Epelman *et al.* 2014; Rőszer 2015). Monocyte-independent proliferation of local macrophages into a novel macrophage phenotype with CXCR3

chemokines receptors has been shown to attract cytotoxic eosinophils to filarial helminths (Turner *et al.* 2018).

In filarial infections, both Th1 / Th2 driven cellular immune responses are activated (Tamarozzi *et al.* 2011; Babayan *et al.* 2012). While the Th1 response activates cytotoxic CD4+ T- lymphocyte inflammatory responses to clear *Wolbachia* released by dead Mf around the worm, Th2 sets in to contain damages to surrounding tissues from persistent Th1 responses by influencing macrophages to release TGF_{β} and IL10 cytokines (Specht *et al.* 2012) responsible for promoting encapsulation and tissue homeostasis (Allen *et al.* 2008). Classical activation of macrophages in onchocerciasis is caused by *Wolbachia* antigen via TLR2 and TLR6 to produce Th1 and Th17 pro-inflammatory responses (Gentil *et al.* 2012). TLR2 forms a heterodimer with TLR6 to initiate a cascade of macrophage activation (Oliveira-Nascimento *et al.* 2012). Excretory and secretory worm products also influence macrophages and monocytes to stimulate vessel growth and alteration of lymphatic function (Weinkopff *et al.* 2014).

The role of macrophages in the pathogenesis of visual impairment and blindness are well-articulated in a review (Tamarozzi *et al.* 2011) and can serve as a typical example of its role during microfilaricidal disease control of larval stages. Macrophages are activated in the presence of *Wolbachia* via TLR-2 not linked to adaptor MyD88/Mal to release pro-inflammatory cytokines that attract neutrophils which release oxidative products (Mylonas *et al.* 2013). CxCR2 are released to direct neutrophils to

the site of infection. Permanent damage to the eyes is caused by the combined actions of macrophages and eosinophils.

2.3.1.4.3 The Role of Neutrophils

Neutrophils are the most abundant innate immune cells and the first granulocytes to be recruited at the site of infection (Delves *et al.* 2011). They are polymorphonuclear (PMN) leucocytes produced in the bone marrow and stored in the circulatory system for about 24 hours but could last up to 5.4 days. As the lifespan of PMN leukocytes is short, they are massively recruited from the bone marrow following an infection. Inactivated neutrophils enter the body tissues where they die by apoptosis after a few days. The structure and functions of human and cattle neutrophils and other immune cells was updated in a published review (Makepeace *et al.* 2012). Mature human neutrophils have two types of toxic granules based on size: the dominant large primary azurophil and smaller secondary specific granules (Bainton and Farquhar 1966). At the pro-granulocyte stage, only azurophils are active. A third granule is present in bovine neutrophils which contains cationic proteins with antimicrobial properties (Gennaro *et al.* 1983).

During the respiratory burst, myeloperoxidase (MPO) encoded by the MPO gene located on chromosome 17, is the major enzyme released from azurophil granules (Delves *et al.* 2011). It is the most abundant protein and toxic enzyme of neutrophils (Schultz and Kaminker 1962). The expression of MPO is induced by granulocyte colony stimulating factor (G-CSF) via GM-CSF and TNF_Y. It utilises hydrogen peroxide to generate hydrogen hypochloride (Harrison and Schultz 1976) and other reactive

oxygen species which kills the pathogen. Antimicrobials such as lysosome hydrolase, defensins, bacteriocidal permeability increasing protein (BPIP), elastases and cathepsin G are also stored in azurophils (McFarlane *et al.* 2008), which constitutes about 5% of the neutrophil's dry weight. The specific neutrophil granules contain lactoferrins and metalloprotease 9 or gelatinase B. The last set of specific granules are actually secretory vesicles containing serum albumin. Based on specific activities of granules, some authors consider neutrophils as having four types of granules (Lacy 2006). Elastases and gelatinases released by azurophils facilitate the migration of neutrophils through the extracellular matrix to the site of infection.

Neutrophils possess numerous PRRs, mostly TLRs and NLRs used to sense PAMPs or DAMPs on pathogens, and damaged or senescent cells with high specificity as earlier reviewed under receptors (Section 2.3.1.4.1.2). Cytokines released by sentinel cells activate neutrophils to become polymorphonucleated and chemokines attract them to the site of infection. On rare occasions, the neutrophil could acquire MHC class II to initiate adaptive immune responses by contributing to antigen processing and presentation. At the site of infection, activated PMN neutrophils produce cytokine IL-8 to increase vascular permeability and recruit more neutrophils (Brattig *et al.* 2001). The mechanism of neutrophil degranulation is mediated via the G-protein coupled (GPC) signal transduction pathway after binding of GPCR to IL-8 or F-Met-Lev-Phe (Lacy 2006). Degranulation results in toxic respiratory burst by exocytosis accompanied by the release of more cytokines to recruit additional neutrophils or M2 macrophages to modulate damage caused to neighbouring tissues. There are four factors that regulate exocytosis. The commonest factors are calcium and

polyphosphorinositol (which is catalysed to phosphatidylinositol 3-kinase (PI3K)-ϒ) levels regulated by calcium signalling and phospholipid signally pathways. Exocytosis promoted by B-arrestin or a group of cytosolic phosphoproteins promotes degranulation of primary and secondary granules, while guanosine triphosphatase (GTP) regulates the degranulation of cytokines or mediators (Lacy 2006).

In the event of overexpression of MPO by neutrophils, as in most autoimmune disorders and chronic diseases, a Th2 adaptive immune response is initiated and vascular permeability increased (Strzepa *et al.* 2017). In helminth infection, tolerance is mediated by CLRs via mannose receptor (Hardison and Brown 2012). However, in filarial diseases, the role of neutrophils in both worm killing and survival has not been fully elucidated but depends on the endobacteria, *Wolbachia* which are released into host tissues during parturition (Kozek 2005), alongside secretions (Landmann *et al.* 2010; Melnikow *et al.* 2011) or as surface proteins via the cuticle (Armstrong *et al.* 2014). Natural death or drug induced death of Mf leads to release of many *Wolbachia* in the skin which are responsible for the pathology observed during infection (Seidenfaden *et al.* 2001; Tamarozzi *et al.* 2011). The principal PAMPs from *Wolbachia* are *W*SP and *w*BmPAL, which are surface membrane proteins and ligands to TLRs on neutrophils. WSP activates TLRs 2/4 and wBmPAL activates TLRs 2/6 to cause inflammation and oedema of affected tissues (Brattig *et al.* 2004). Because worm tissues cannot be engulfed by phagocytes, neutrophils accumulate around them and their lifespan is extended by WSP (Brattig *et al.* 2001; Hansen *et al.* 2011). A cross section of a healthy *Onchocerca* nodule will portray the worm(s) surrounded by concentric rings of neutrophils (Brattig *et al.* 2001; Hansen *et al.* 2011) and a few

eosinophils not very close to the worm surface (Wildenburg *et al.* 1996). This relationship may be responsible for the longevity of the worms.

2.3.1.4.4 The Role of Eosinophils

Eosinophils are granulocytes with a bi-lobed nucleus. They are potent innate immune cells against multicellular organisms, fungi and viral infections. Eosinophils are activated by IL-5 (Abraham *et al.* 2004) and attracted to the infected site by RANTES or eotaxin (Pearlman *et al.* 1999). Degranulation and the release of toxic substances at a target via micro-tubules requires direct contact with the invader (Klion and Nutman 2004). Activated eosinophils also release cytokines to stimulate mast cells to release histamines amongst other toxic substances (Urb and Sheppard 2012). The histamines released are responsible for pruritus (Remme 1995) and/or sclerotic keratitis (Wildenburg *et al.* 1994). The scratching and wounding of the itchy skin predispose the skin to secondary bacterial infection. After treatment with IVM (O'Connell *et al.* 2011) or DEC, there is increase in IL-5 within the lymph nodes and eosinophilia before the appearance of the symptoms (Klion and Nutman 2004). A dead onchocercoma contains dead calcifying worms, active inflammation, macrophages, giant cells and eosinophils adhered to the degenerating cuticles of Mf (Brattig NW *et al.* 1991). In chronic inflammation, disintegrating Mf and neutrophils were encapsulated within an abscess (WHO-Update 1981; Trees 1992; Johnson *et al.* 1995). There seem to be some correlations between female worm gravidity with intra-uterine Mfs and eosinophils (Seidenfaden *et al.* 2001). In the early infection, the blood eosinophil level increases (Wildenburg *et al.* 1994) but as the worm matures, it decreases. The main granule of the eosinophil contains major basic proteins (MBP)

(Plager *et al.* 1999) capable of immobilising Mf of all the larval stages but not L4 and active unproductive adult *O. volvulus* (Strote *et al.* 1990). There is sufficient evidence to show that eosinophils can immobilise Mf of all the larval stages except L4 (Wildenburg *et al.* 1996). For eosinophils to effect worm killing, they need to adhere to the parasite's cuticle (Melo and Weller 2010) and degranulate toxic substances such as eosinophil cationic protein and eosinophil peroxidase onto the parasite (Hansen *et al.* 2011). Eosinophils appear to be much more efficient when their target is immobilised within lymph nodes (Hoerauf *et al.* 2005).

2.3.1.5 *Adaptive Immunity*

Resting T-lymphocytes play a pivotal role in adaptive immunity, during which a specific encounter with an infection is tackled by effector cells (Zhang and Bevan 2011) and memorized by B cells (Harris and Gause 2011) in readiness for an anamnestic response on a second occurrence (Isobe *et al.* 1986; Andersen *et al.* 2006). T lymphocytes (T cells) are white blood cells produced in the bone marrow but matured in the thymus glands or in tonsils, while B lymphocytes (B cells) mature in the bone marrow of mammals (Harris and Gause 2011) or Bursar of Fabricius of birds. Upon stimulation, naïve T cells split into many subsets of cells depending on the antigen identified. The main T cell subsets are T helpers (Th), T (cytotoxic) killers and T regulatory cells (Berger 2000). T helper cells, also known as T4 cells or $CD4^+$ cells because of the presence of the $CD4^+$ glycoprotein molecule on its surface, interacts with antigen presented by MHC class II to produce co-stimulants to activate macrophages (Rudd *et al.* 2010) and other effector cells, help suppress or regulate

immune responses, and cause maturation of B-cells into plasma and memory B cells. $CD8^+$ cells or T killer cells are cytotoxic to cells infected with viruses or other intracellular pathogens and tumours (Zhang and Bevan 2011) when ligated to MHC class I molecules in the presence of a co-stimulus (Andersen *et al.* 2006). T cell-restricted immune responses are specific and follow different pathways depending on the molecular biology of the antigen (Berger 2000). In filarial infections, the ratio of Th1- and Th2- lymphocytes determine the fate of the parasite (Babayan *et al.* 2012; Gentil *et al.* 2012). *Wolbachia* surface proteins stimulate a Th1- proinflammatory response (Brattig *et al.* 2004) that tends to protect the worm (Hansen *et al.* 2011). The activation of CXCRs on macrophages by IL-4 mediate Th2 responses and eosinophil degranulation (Turner *et al.* 2018). Numerous experiments on mice and jirds have affirmed the role of T- lymphocytes in the effective control of skin Mf (Carlow Clotilde *et al.* 1988; Allen and Maizels 2011; Harris and Gause 2011; Gentil *et al.* 2012).

2.3.2 Mechanisms of Immune Evasion by Filarial Parasites

The penetration of parasites into the vertebrate host is met with an innate immune response from sentinel cells. However, filarial parasites survive within vertebrate hosts for years (as long as 10 to 15 years in onchocerciasis) due to immunomodulatory effects. In laboratory models, significant number of L3s of *L. sigmodontis* (Goff *et al.* 2000) and *L. loa* (Wanji *et al.* 2002) escape aggressive immune attack within body tissues upon invasion by entering into the lymphatic vessels (Babayan *et al.* 2003; Bain and Babayan 2003), while in *O. volvulus*, the L4 stage takes advantage of its morphological architecture of a distinctive cuticle to stop eosinophil

adherence (Brattig *et al.* 1994; Selkirk *et al.* 1998). The principal challenge for the survival of adult worms is the ability to resist the oxidative environment created by the neutrophil respiratory burst and eosinophil degranulation onto the worm cuticle (Brattig *et al.* 1994; Selkirk *et al.* 1998). *B. malayi* synthesizes glutathione peroxidase to protect its cuticle from the damaging effects of free oxygen radicals by reducing phospholipid hydroperoxides (Tang *et al.* 1995). Glutathione peroxidase is regulated independently from selenium which is incorporated as vitamin E into worm cuticle from the host's extracellular fluid (Selkirk *et al.* 1998). Other antioxidants such as thioredoxin peroxidase, are expressed in most stages of *O. volvulus* (Lu *et al.* 1998; Dzik 2006) and in nodule fluid of *O. ochengi* (Armstrong *et al.* 2016), and are highly up-regulated.

Another mechanism of immune evasion is by active secretion of homologues of anti-inflammatory cytokines, such as macrophage migration inhibitory factor (MMIF) which was observed in *B. malayi*, *W. bancrofti* and *O. volvulus* and shown to be capable of inhibiting up to 75% of monocyte and macrophage migration, thereby affecting macrophage chemotaxis in an identical way to human MMIF (Pastrana *et al.* 1998). Other major cytokine homologue secreted by macrophages such as TGF-β and galectins (González-Miguel *et al.* 2015) were isolated from nodule fluids obtained from *O. ochengi* (Armstrong *et al.* 2016). There are other immune modulatory products including ALT2 reviewed under vaccine candidates whose contribution to the prolongation of worm lifespan have been targeted for vaccine research (Anand *et al.* 2011; Sam *et al.* 2011).

The last category of immune evasion mechanisms is the principal benefit of mutualism between the worm and its endosymbiont bacteria, *Wolbachia*, in its nodule-dwelling hosts, *O. volvulus and O. ochengi*, characterized by the neutrophilic "protective coating" on the viable worm's surface (Tamarozzi *et al.* 2016). *Wolbachia* attract neutrophils to the worm's immediate surroundings and increases its lifespan. Infiltrated neutrophils form extracellular traps (NET) around the worm to contain the spread of cytotoxic substances into host tissues. By so doing, the neutrophils, through an unknown mechanism, contribute to the enhancement of a state of tolerance of the worm (Brattig *et al.* 2001). Thus, the mutualism extends the worm's lifespan. *Wolbachia* is absent in *O. flexuosa,* a sessile nodule-forming filaria of deer that does not attract neutrophils (Plenge-Bönig *et al.* 1995). Adult *O. flexuosa* lives for only about 18 months while surrounded by eosinophils (Wildenburg *et al.* 1996). Antibiotic-treated worms after neutrophil depopulation do not usually survive for more than a year using the cattle model (Langworthy *et al.* 2000; Gilbert *et al.* 2005; Nfon *et al.* 2006; Hansen *et al.* 2011), or up to two years after doxycycline therapy in humans (Hoerauf *et al.* 2008b; Walker *et al.* 2015).

Chapter 3 Efficacy of Combining Immunotherapy with Mutated Onchocystatin and Sublethal Oxytetracycline Chemotherapy on *O. ochengi* in Cattle

3.1 Introduction

Antibiotic of the tetracycline family was first shown to have a lethal anti-filarial effect on a cattle filarial worm, *Onchocerca ochengi* (Langworthy *et al.* 2000; Gilbert *et al.* 2005). Treatment is successful only when the antibiotic is administered intermittently over six months, as in cattle, or as a 4 - 6 week continuous daily regimen in humans (Hoerauf *et al.* 2008b). Macrofilaricidal effects depend on abrogation of the relationship between the filarial host and its endosymbiotic bacteria, *Wolbachia* (Langworthy *et al.* 2000). *Wolbachia* is found within host cells of the hypodermal cord and the female reproductive tracts (Bandi *et al.* 1999; Hoerauf *et al.* 1999; Langworthy *et al.* 2000; Gilbert *et al.* 2005). *O. volvulus*, the lone causative organism of human onchocerciasis, will not complete its lifecycle in species other than humans and great apes. So, *O. ochengi*, its closest biological relative (Morales-Hojas *et al.* 2006), has been an exemplary experimental model for pharmacological research on onchocerciasis (Trees 1992; Trees *et al.* 2000). Antibiotics of the tetracycline family function by interfering with protein synthesis and therefore have the ability to penetrate cell membranes to target the ribosomes (Chopra 1994). Previous studies monitoring the post-therapeutic depletion of *Wolbachia* from *O. ochengi* nodules revealed that the bacteria were eliminated from all worm histological sections at 24 weeks after the onset of treatment, when the last monthly dose of adulticidal

oxytetracycline therapy (ADT) was administered (Gilbert *et al.* 2005). Meanwhile, with sub-lethal therapy (SLT), a few months after oxytetracycline chemotherapy terminated, *Wolbachia* was still present within the ovaries (Gilbert *et al.* 2005; Nfon *et al.* 2006). In the human trial protocols, depletion of *Wolbachia* from *O. volvulus* was first evident after 24 weeks and the bacteria were cleared from most of the worms at 40 weeks after onset of treatment (Hoerauf *et al.* 2008b). The exact mechanism of worm death after the depletion of *Wolbachia* has not been clearly elucidated.

In the cattle model, worm death has been attributed to immunological changes associated with the replacement of neutrophils by activated eosinophils around the worm surface (Nfon *et al.* 2006; Hansen *et al.* 2011) resulting from the sustained removal of *Wolbachia* endobacteria during the ADT regimen (Langworthy *et al.* 2000). Depletion of *Wolbachia* endobacteria after ADT antibiotic therapy in cattle, lead to worm death within 52 weeks (Makepeace *et al.* 2006) while continuous daily oral doxycycline administration in humans cause adult worm death 24 to 36 weeks post treatment (Hoerauf *et al.* 2008b). In humans or small animal models continuous doxycycline administration resulted in embryostasis, sterility (A. Hoerauf *et al.* 2000a; Hoerauf *et al.* 2003; Turner *et al.* 2010), cellular apoptosis, and a sustained drop in microfilarial load (Landmann *et al.* 2011) before death of worms long after therapy ended (Hoerauf *et al.* 2008b).

The potential use of antibiotic therapy in control programs will greatly be enhanced if the duration of treatment is shortened. However, studies to identify other potential antibiotics such as chloramphenicol and ciprofloxacin (Achim Hoerauf *et al.* 2000) or

macrolides such as azithromycin (Hoerauf *et al.* 2008a) with superior macrofilaricidal effects to the tetracyclines were initially unsuccessful. Thus, rifampicin used to treat tuberculosis was no more effective than tetracyclines in treating onchocerciasis in humans (F.O. Richards *et al.* 2007; Specht *et al.* 2008) and in cattle (Bah *et al.* 2014). The availability of tools such as *Wolbachia*-infected insect cell-lines and small animal filarial models has enabled the evaluation of novel antibiotics, *e.g*., corallopyronin A, for efficacy against *Wolbachia* (Schiefer *et al.* 2012). *Wolbachia* cell-lines have also been used to test and repurpose existing drugs belonging to the tetracycline, fluoroquinolone, and rifamycin families (Johnston *et al.* 2014; Taylor *et al.* 2014). Nonetheless, the development of a vaccine against filarial diseases remains a complementary or alternative option for the eradication of onchocerciasis.

Antibody-mediated cytotoxicity (ADCC) is one of the immune mechanisms employed in the control of helminth infections (Allen and Maizels 2011; Harris and Gause 2011). B lymphocytes are stimulated by Th2 lymphocytes to produce antibodies against specific antigens present on the worm surface (Paul and Zhu 2010). When these cytotoxic antibodies are produced and the surface of the parasites is coated with them, the ability of larvae of *H. polygyrus bakeri* to penetrate gut mucosa and the migration of adults are affected (Harris and Gause 2011). The number of bonds and strength of the links between antibody and the immune cells mediating cytotoxicity such as type 2 immune cells (eosinophils, basophils, mast cells, or macrophages) improves the efficiency of ADCC. The Fc receptors of the effector immune cells couple with the Fc portions of antibody complexes (IgE, IgG1, IgG3 or IgM) already coated on worm surfaces to activate release of toxic granules (David *et al.* 1980).

Putative immunes (PI) and treated patients living in filarial endemic areas have high serum IgG isotypes and IgE (Soboslay *et al.* 1997). Specifically, high IgG3 levels are larvicidal *in vitro* and may be responsible for PI status in human (Amambua *et al.* 2006). Filarial nematodes, in order to survive in the definitive host, have evolved mechanisms that interfere with surface antigen presentation to immune cells (Allen and Maizels 2011). One major protein involved in worm protection is a cysteine protease inhibitor (CPI), or cystatin (Gregory and Maizels 2008), which acts as an immunomodulator (Pfaff *et al.* 2002). Cystatins are present in both animals (Murray *et al.* 2005) and plants (Gregory and Maizels 2008). Onchocystatin is a cystatin from *Onchocerca* species, which is also known as Ov7 or CPI-2 (Lustigman *et al.* 1992). Its presence on the cuticle of adult female worms and most other stages of *O. volvulus*, except mature Mf (Lustigman *et al.* 1991), makes it a potential vaccine target. Onchocystatin also plays an important role in parasite development by arresting the activity of cysteine proteases at the end of each moult (Gregory and Maizels 2008). Onchocystatin also has a role in immune evasion (Pfaff *et al.* 2002), blocking the major histocompatibility complex (MHC) class II site on the antigen presenting cells (APC) and their ability to process antigen via papain-like proteases and asparaginyl endopeptidase (Villadangos and Ploegh 2000; Manoury *et al.* 2001).

Cytophilic antibodies (IgG3) generated by PI target onchocystatin, retard larval development and affect worm viability, and thereby could potentially play a vital role in the long term control and prevention of onchocerciasis (Cho-Ngwa *et al.* 2010). Vaccines produced against mutated onchocystatin (LsCPI) and abundant larval transcript-1 (LsALT) induced significant prophylactic protection in the *L. sigmodontis*

mouse model when co-administered with Th2 enhancers (Babayan *et al.* 2012) and in the gerbil (Arumugam *et al.* 2014a). Mutation of a key asparagine residue in CPI-2 made the vaccine highly immunogenic (Murray *et al.* 2005). However, gerbils vaccinated with unmodified *Bm*CPI-2, failed in preventing new infections even though there were post vaccination changes in the adult worm migration pattern (Arumugam *et al.* 2014b).

In this experiment, we tested for the first time the hypothesis that immunotherapy with mutated *Oo*CPI-2 in cattle prior to sub-lethal chemotherapy might enhance the efficacy of this relatively short antibiotic treatment.

3.2 Materials and Methods

3.2.1 Ethical and Safety Considerations

The Ethics Committee and the Regional Programmes Committee of the Institute of Agricultural Research for Development (IRAD), Wakwa Regional Centre authorized the fieldwork of this study. All procedures performed on the experimental cattle in Cameroon were mild in severity and were equivalent to those authorized by a Home Office Project License on Animals (Scientific Procedures) Act, 1986 for experimental infections of cattle in the United Kingdom. All laboratory procedures were conducted according to the safety norms put in place by the various laboratory safety officers.

3.2.2 Materials

3.2.2.1 *Experimental Animals*

Thirty-Five Ngaoundere Gudali zebu cattle (Figure 3.1, *Bos indicus*) of good body condition scores (BCS ~ 3.5/5) were purchased from local markets across the Vina Division for the experiment. Each animal harboured at least 24 intradermal *O. ochengi* nodules and was confirmed free from other skin disorders such as dermatophilosis and *Demodex* infestation before recruitment based on clinical examination.

3.2.2.2 *Experimental Site*

Sampling of the experimental cattle was carried out at IRAD Wakwa (Figure 3.1). The sampling pen was located less than 100 m from the Wakwa Veterinary Research laboratory. This facilitated the rapid and immediate transfer of samples to the laboratory for preliminary analysis or processing for storage after collection. The transmission rate of *O. ochengi* at this site (Figure 3.1) is negligible (Achukwi *et al.*

2000; Tchakouté *et al.* 2006), as previous vaccine trials requiring similar environmental conditions reported no new infections (Njongmeta *et al.* 2004; Bronsvoort *et al.* 2005; Achukwi M *et al.* 2006; Makepeace *et al.* 2009).

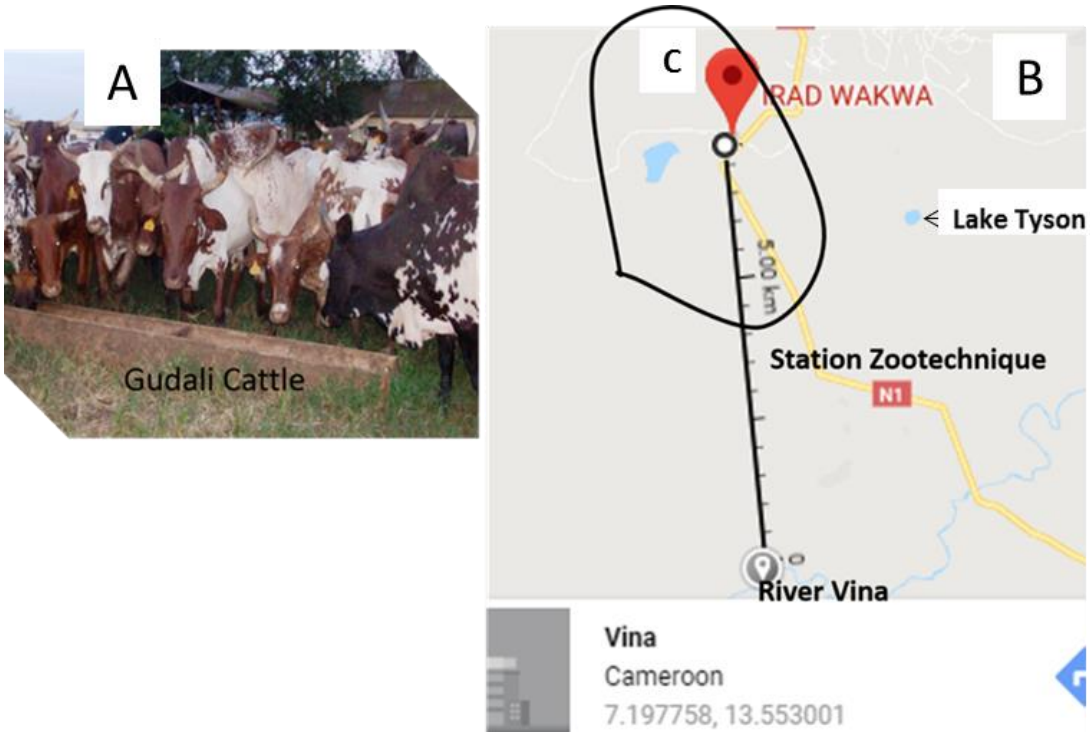


Figure 3.1: A. Ngaoundéré Gudali Zebu cattle used for the experiment. B. Extract of google map of IRAD Wakwa showing the grazing zone of the experimental herd (C-black circle) in relationship to River Vina (5km) where the *O. ochengi* transmission rate is high. N1 National road no. 1.

At IRAD Wakwa, research activities are routinely being conducted on livestock, agriculture, and animal health in the High Guinea Savannah climate, corresponding to Cameroon's agro-ecological zone II (Figure 3.2). It is situated some 10 km from Ngaoundéré Metropolis and the nearby Wakwa village plays an important role in the dissemination of agricultural research results in the Adamawa Region of Cameroon located between Latitude 7° 19' 39 N and Longitude 13° 35' 4 E (Figure 3.1). Wakwa

is 1,000 m above sea level and has an annual rainfall of 1,000 to 2,000 mm (Figure 3.2) with temperatures varying between +10 and +35 ^{o}C. The cattle grazed within a perimeter of 4-kilometre radius from their sleeping point (Figure 3.1).

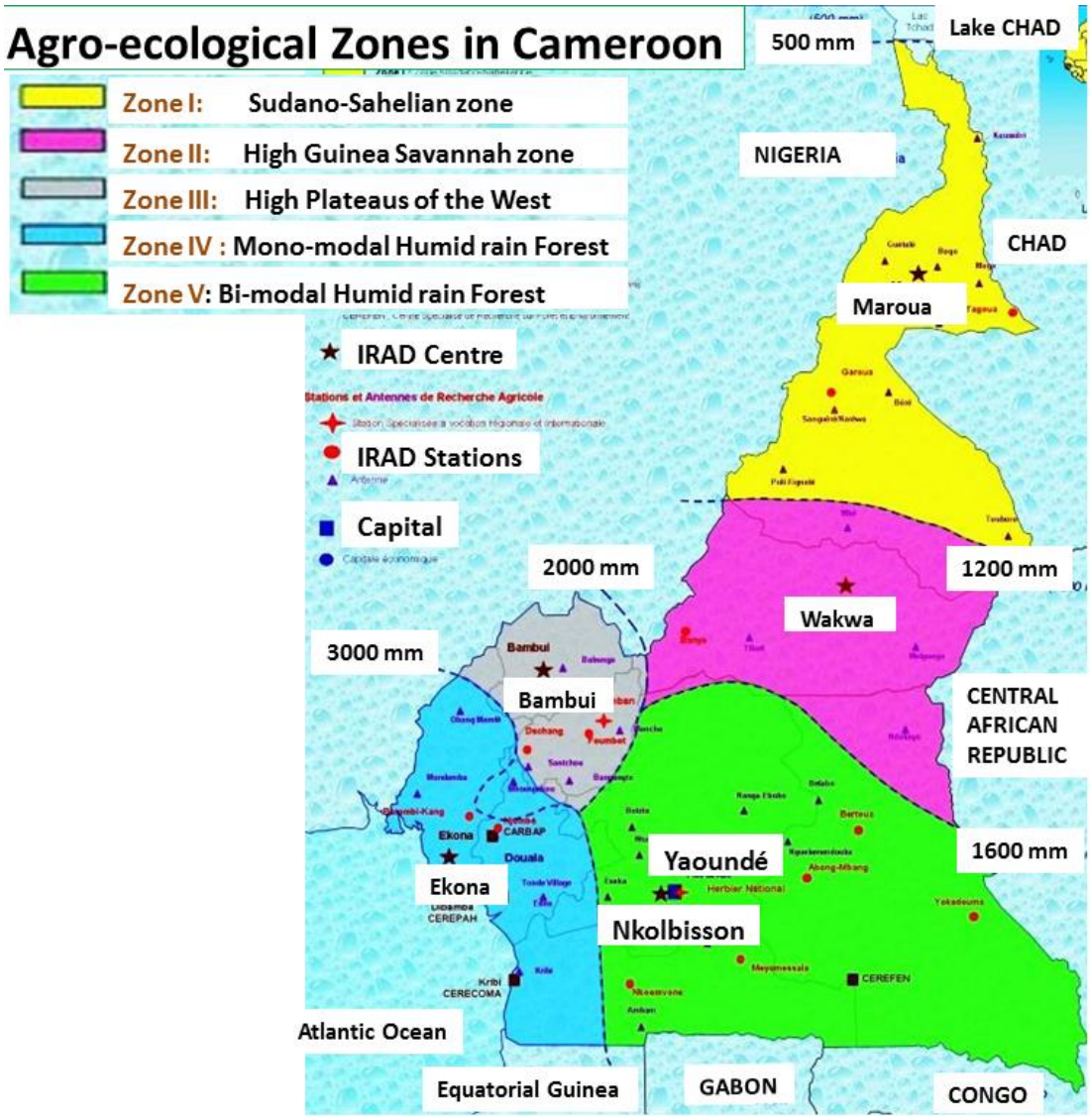


Figure 3.2: Agro-ecological map of Cameroon. The Wakwa Research Centre is made up of two Stations (Wakwa and Banyo) and four antennae (Mbe, Meiganga, Tibati and Ndokayo). The values in mm at the limits to agro-ecological zones refer to annual rainfall.

3.2.2.3 *Health Care Management of Experimental Cattle*

3.2.2.3.1 Vaccination

The recruited cattle were vaccinated against Black Quarter's Disease and Haemorrhagic Septicaemia using Symptovax® and Pasteurellovax, ® respectively. The vaccines were produced by the National Veterinary Laboratory of Cameroon (LANAVET). The animals were observed for a month before the start of the experiment.

3.2.2.3.2 Preventive Therapy

During the stabilisation period, all the cattle were treated with diaminazine aceturate (SANGAVET®) against trypanosomosis, an endemic disease condition. SANGAVET is also active against babesiosis. In February 2012, after the time-point at 36 weeks (T36), all the animals were given intramuscular doses of vitamin AD_3E (3 ml per 100 kg body weight) to stabilise them during the extreme dry season. Tick control was routinely carried out by weekly spray with alpha cypermethrin (Butox®) during the rainy season or fortnightly during the dry season. Our sampling techniques included surgical extirpation of nodules. The sutured wounds were protected from flies and potential myiasis by the application of Battles Summer Fly Cream™.

3.2.2.3.3 Handling of Infections During Experiment

Two cases of abortions occurred within the second month of the experiment. Cow 232 of the onchocystatin immunochemotherapy (OVT) group aborted shortly after receiving the sub-lethal oxytetracycline regimen. This cow received no further post-abortion treatment. However, when cow 233 of the control group aborted, it was administered a procaine penicillin marketed as Combikel® (20 ml deep

intramuscularly). The causes of the two abortions were not determined. Towards the end of the experiment, the cows 209, 211, 222, 227 and 231, which were highly emaciated with a BCS less than 2.5, were de-wormed with levamisole boluses (7.5 mg/kg) in February 2012.

3.2.2.3.4 Husbandry and Feed Supplementation

The cattle were grazed on *Bracharia ruziziensis, Stylosanthes guianensis* and natural grass paddocks everyday between 9:00 AM and 6:00 PM. During the day, the herdsman monitored the cattle. At night, they were confined at the Centre's sleeping pen and guarded by a night watchman who ensured that the animals did not escape out of the 3 – 4 km safe perimeter ring to graze. While grazing, the cattle had contact with other animals of the Centre and of the neighbourhood.

During the dry season, characterised by feed shortage due to bush burning, supplementary hay produced mainly from *Bracharia ruziziensis* and *Stylosanthes guianensis* were fed to the cattle. In addition, a maintenance ration of cotton seed cake (500 g per cattle per day) was fed once daily between 7:00 and 9:00 AM to all cattle. To ensure that the weaker animals had equal access to feed, the cattle were fed in five sub-groups with the weaker animals grouped together. Salt was available *ad libitum*. During the rest of the day, the cattle grazed on the degraded paddocks and along streams free from black flies.

3.2.3 Methodology

3.2.3.1 *Experimental Design*

A randomised repeated measure experimental design was set up with 5 experimental groups of 7 cattle each and parameters collected at 6 predetermined time-points (T)

(Table 3.1). Randomization of age-stratified cattle by ear tag was performed with the aid of random numbers on a scientific calculator. The four treatment groups had immunochemotherapy (OVT) as the main test group; immunotherapy (OVC) as the vaccine control; Adulticidal therapy (ADT) as the positive antibiotic control and the sublethal antibiotic therapy (SLT) as the negative antibiotic control. The fifth group was untreated. Data were collected one week before antibiotic treatment only from the vaccinated groups. At the commencement of treatment (T0), then at 4, 8, 12, 36 and 52 weeks, data were collected from all the groups. The treatments administered were vaccine and / or antibiotics (Table 3.1).

Table 3.1: Characteristics of experimental groups and treatment regimens

Group (abbreviation)	Sample		Mean Age	Body Weight	Treatment
	Males	Females	(years) ± SD	(Kg ± SD	
Untreated control (CON)	1	6	6.2 ± 3.7	268 ± 44	None
Sub-lethal antibiotic	1	6	5.7 ± 2.3	283 ± 48	Oxytetracycline 10 mg/kg IV, daily
therapy (SLT)					daily for 14 days
Adulticidal antibiotic	0	7	5.9 ± 1.6	268 ± 25	Oxytetracycline 10 mg/kg IV, daily
therapy (ADT)					for 14 days; then 20 mg/kg IM,
					monthly for 5 months
Onchocystatin vaccine	1	6	7.1 ± 3.5	300 ± 43	0.5mg recombinant onchocystatin;
control (OVC)					50% IM, 50% SC
Onchocystatin vaccine with	1	6	6.4 ± 2.5	261 ± 40	0.5mg recombinant onchocystatin;
antibiotic therapy (OVT)					50% IM, 50% SC; 7 day pause, then
					oxytetracycline 10 mg/kg IV, daily
					for 14 days

IV, intraveneous; SC, Subcuteneous route; SD, Standard deviation.

3.2.3.2 *Onchocystatin Vaccination of Experimental Groups*

Two experimental groups (14 cattle) were destined for vaccination. Both groups were vaccinated a week before the start of chemotherapy. For inoculation into cattle, 2.5 ml (7.5 mg) of the recombinant mutated onchocystatin (produced by Creative Biolabs, Shirley, NY, USA) was diluted in 12.5 ml sterile Dulbecco's PBS (Sigma) and

formulated in 15 ml alum adjuvant (Imject, Life Technologies, Paisley, UK) at a 1:1 ratio to have 14 1-ml doses of 0.5 mg each. The alum adjuvant was thoroughly mixed by shaking, before adding to the vaccine drop-wise while gently swirling the receptacle with the other hand. The mixing was done over a period of 30 minutes before fourteen 2-ml syringes were loaded and protected in sheaths. All procedures were carried out in a sterile hood. The loaded syringes were then immediately transported to the field for vaccination as indicated in Table 3.1.

3.2.3.3 *Antibiotic Chemotherapy*

The OVT, SLT and ADT groups received intravenous injection of oxytetracycline (Terramycin® LA, Zoetis, London, UK) chemotherapy (SLT) at T0. The ADT group was then followed up with 5 monthly intermittent doses of Terramycin® LA from T4 onward (Table 3.1). Previous studies showed that ADT therapy (Table 1) killed 80 % of treated worms in 52 weeks (Gilbert *et al.* 2005) with important changes occurring at T8, T12, T36 and T52 (Nfon *et al.* 2006; Hansen *et al.* 2011).

3.2.4 Specific Experimental Designs

To minimise cost, subsets of the samples were processed to respond to different specific objectives of the study (Table 3.2).

3.2.5 Production, Preparation, and Application of Recombinant Vaccine

The mutated onchocystatin nucleotide sequence was synthesized using the *O. ochengi* (*Oo*CPI-2) protein sequence published in GenBank ACB70196.1 (Makepeace *et al.* 2009) as the basis for codon optimisation and cloned in *Escherichia coli* by a contract research organisation (Creative Biolabs, Shirley, NY, USA). Mutation of onchocystatin was achieved by replacing the amino acid, asparagine (required for

enzymatic activity) located at position 44 of the published wild-type sequence with leucine. Leucine is a non-acidic residue that enhances immunogenicity (Babayan *et al.* 2012). Unlike the *Oo*CPI-2 sequence, this critical residue is located at position 66 in the *L. sigmodontis* and position 78 (Figure 3.3) in the *B. malayi* cystatin orthologues (Nfon *et al.* 2006), respectively, because of the variable N-terminal extension in these proteins.

Table 3.2: Specific objectives and analytic techniques.

Objectives	**Analytical techniques (Groups)**	**Time-points analyzed**	**Result Chapter**
Onchocystatin vaccination response	Determination of IgG level by ELISA (OVC, OVT and CON groups)	T-1, T0, T4, T8, T12 and T36	One
Immuno-Chemotherapeutic effects on worm viability	Parasitology (CON, SLT, ADT, OVC and OVT)	T0, T4, T8, T12, T36 and T52	One
Immuno-Chemotherapeutic effects on nodule eosinophilia	Histology, Geimsa stain and eosinophil count with Zeiss image microscopy (SLT, ADT, OVC and OVT)	T0, T12 and T36	One
Antibiotic Chemotherapeutic effects on female worm proteome	Extraction and purification of soluble proteins; MS-LC protein quantification and analysis of data by Progenesis (CON, SLT and ADT).	T0, T12 and T36	Two
Antibiotic Chemotherapeutic effects on nodule gene expression	RNA extraction and purification, RNA sequencing and transcriptomics.	T4, T8 and T52	Three

The synthetic construct was cloned into the pET-30a (+) vector (EMD Millipore, Billerica, MA, USA) and expressed as a 41 kDa *Schistosoma japonicum* glutathione S-transferase fusion protein with a histidine tag in *E. coli* strain BL21 (DE3). Soluble recombinant GST-onchocystatin was recovered at a purity of >90% using nickel-nitrilotriacetic acid agarose resin, and endotoxin was reduced to a level of <0.1 EU/ml with the ToxinEraser Advanced Endotoxin Removal Kit (GenScript, Piscataway, NJ, USA). Initially, 1 mg of the mutated *Oo*CPI-2 vaccine was produced, tested in mice by

Dr Simon Babayan in Edinburgh and confirmed to be immunogenic before scaling-up to 30 mg, from which a portion was used during this field trial (Table 3.1).

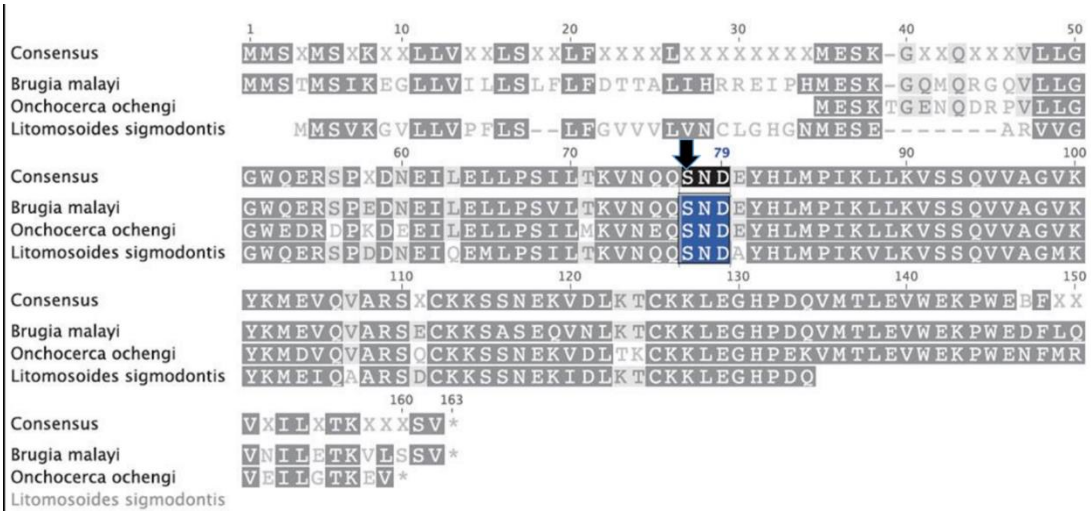


Figure 3.3: Onchocystatin sequence. Arrow indicates location of the critical residues for enzymatic activity corresponding to position 44 in *O. ochengi* and 66 in *B. malayi.* Figure kindly provided by Dr Simon Babayan.

3.2.5.1 *Sampling Techniques*

At each time-point, due to the large sample size, sampling was done from 6 AM to 3 PM and spread over three days. The vaccine treated (OVT) and ADT animals were sampled on day 1; SLT and vaccine control (OVC) on day 2, and the CON group on day 3. The animals to be sampled each day were confined in a specially constructed pen with a crush through which the animal passed to enter a roofed enclosure for sample collection.

3.2.5.1.1 Animal Restraint During Sampling

Each animal was restrained in lateral recumbency, with the hind limbs tied wide apart and one fore-limb attached to the horn and fixed to a pole (Figure 3.4). The sampling

procedure on each animal lasted 15 minutes. After sampling, the tail was passed between the thighs and held firmly by an assistant as the hind limbs, forelimbs and the head were released before it was helped to stand up.

Figure 3.4: Experimental bull. Restraint position for identification of nodules from the scrotal area encircled towards the flanks (A) and within the thighs (B). The same posture is used during nodulectomies of nodules (C) tattooed at three points.

3.2.5.1.2 Nodule Identification

On lateral recumbency and the hind limbs wide apart (Figure 3.4), nodules were searched for from around the udder (females) or scrotum (males), then outward laterally to the flanks and inner thighs, distally to the tail and anteriorly to the umbilicus and chest regions (arrows A, B on Figure 3.4). An archetypal *O. ochengi* nodule is usually compressible but firm, spherical and intradermal. Hard and/or

discoloured nodules were avoided, as these are signs of natural worm death and calcification. The 24 nodules considered with high confidence to be of *O. ochengi* origin were tattooed (C) after shaving with a clipper. The tattooing was by intradermal injection of approximately 0.5 ml of veterinary tattoo ink (Cox®) at 3 equilateral points around each nodule using an 18-gauge needle. The location of each tattooed nodule was mapped and labelled on a sex-specific "hide map". Nodule clusters were excluded from the studies as they can be difficult to isolate from one another during surgery. Digital video clips of mapped nodule positions were also recorded.

3.2.5.1.3 Blood Collection

Jugular blood was collected into sterile, 10-ml red-capped (additive-free) Vacutainer tubes via 18G Vacutainer needles. The collected blood samples were allowed to clot at ambient temperature for at least an hour, and then refrigerated at 4^{o}C overnight before serum extraction using centrifugation at 3,000 rpm for 5 minutes. The sera were deep frozen in two aliquots at -80^{o}C.

3.2.5.1.4 Skin Snipping

Three skin biopsies weighing ~3mg each were collected from each animal at positions A, B and C along the mid-ventral abdomen (Figure 3.5) into separate 1.5 ml Eppendorf tubes containing sterile RPMI 1640 media (Sigma). The media was supplemented with 200 IU/ml penicillin and 200 µg/ml streptomycin (Sigma). Before snipping, the sampling sites were first swabbed with 70% ethanol and shaved with a blade.

Figure 3.5: Collection of skin snips. A, B and C are standardised locations for skin snipping. The sampling sites were first swabbed with 70% ethanol and shaved with a blade before snipping of a portion of the skin.

3.2.5.1.5 Nodulectomy

Two of four nodules selected by prior randomisation of all nodule locations excised at each time point were used for viability studies. The hide maps or digital video clips were used to confirm nodule location before removal. The surrounding skin was clipped, shaved and disinfected with 70% ethanol. Local anaesthetic (1% lidocaine; Willcain®, Dechra Veterinary Products) was injected intradermally in the tattooed area (Figure 3.5, c) before nodulectomy. The excised intradermal nodules were immediately transported to the laboratory in 7-ml bijou tubes containing sterile phosphate buffered saline (PBS, Sigma) by one of the field assistants for processing.

Each wound was closed with chromic catgut, and summer fly cream (Battle, Hayward & Bower Ltd) was applied to protect from flies, hence preventing myiasis. The sutures were removed ten days after nodulectomy.

3.2.6 Parasitological Analyses for Worm Viability

In the laboratory, the nodules were carefully extracted from excess dermal tissues with a round-bellied surgical blade. Undamaged nodules were processed for worm viability studies. The diameter was measured across two perpendicular planes with a pair of Vernier callipers and the average value recorded. The second nodule reserved for histopathology was injected with a portion of 10% neutral buffered formalin (NBF) using an insulin syringe and stored submerged in the formalin at room temperature.

3.2.6.1 *Determination of Skin Mf Density*

The skin snips were incubated at 37^{o}C for 48 hours. Released Mfs were counted three times at 6, 24 and 48 hours from the medium under a stereo microscope. Fresh RPMI medium was added to the skin snip after each count. During the third refill (day 2), 0.5% collagenase was added to the sterile RPMI 1640 medium and the snips weighed on an analytical balance (Acculab Atilon®). During Mf counting the species observed were recorded (*O. ochengi, O. gutturosa, O. dukei, O. armillata* and/or others) as previously described (Naessens *et al.* 1988).

3.2.6.2 *Nodule Dissection*

The incision in the nodule capsule, made with a scalpel blade, was carefully widened to expose the female worm mass, which was gently pulled out with a pair of forceps or bluntly scrapped onto a glass slide for identification, ageing and determination of motility. It is very easy to distinguish between male and female *Onchocerca* worms,

as the females are much larger than the males. Male worms are highly mobile and can be easily extracted from the coiled female worm mass. Using forceps, they were placed into a 24-well tissue culture plate containing sterile RPMI 1640 medium.

3.2.6.3 *Determination of Worm Motility (viability)*

The entire male worms and cut heads of female worms were incubated for 30 minutes at 37^{o}C in sterile RPMI 1640 medium using tissue culture plates (Nunc, Roskilde, Denmark). Motility was observed under low-powered magnification on a CETI® dissecting microscope and scored on a three-point (0, 1 or 2) scale as previously described (Nfon *et al.* 2006; Hansen *et al.* 2011). Worms with zero motility score were assumed to be dead, and those with scores of 1 (sluggish/moribund) or 2 (normal activity) were viable. The heads of the female worms were returned to the rest of the body after this evaluation.

3.2.6.4 *Determination of Worm Age and Fecundity*

The worms were aged as young, medium, or old based on the complexion of the cuticle. Old worms were brownish-orange in complexion, medium-aged worms were lighter (yellowish), while the young worms were smaller and milky in complexion.

The female worm was removed from the nodule capsule, placed on a depression slide containing a drop of PBS, and carefully loosened to expose all worm segments with the aid of a pair of smooth tip forceps and a needle. The worm was examined under a NIKON SMZ800® dissection microscope from the head to the tail to estimate the gravidity of the uteri on a four-point scale (0, 1, 2 and 3). Depending on the proportion of the uteri filled with motile larvae, non-gravid worms had zero fecundity, while

worms with less than half, two-thirds, or fully gravid uteri were scored as 1, 2 or 3, respectively.

3.2.7 Serology

3.2.7.1 *The Determination of IgG Response to* O. ochengi *Onchocystatin*

Antibody responses to onchocystatin were determined using an ELISA protocol developed from an existing isotype-specific bovine IgG assay (Langworthy *et al.* 2000; Bronsvoort *et al.* 2005), with modifications (only total IgG was measured). Prior to running the test, antigen, serum and conjugate concentrations were optimised by checkerboard titration using pooled *O. ochengi* positive bovine sera (n = 5) from a previous experiment (Gilbert *et al.* 2005) and a negative serum sample from a British cow. Optimal conditions comprised a coating concentration of 5 µg/ml mutated onchocystatin, a test serum dilution of 1/50 and a monoclonal anti-bovine IgG [clone IL-A2, horseradish peroxidase (HRP) conjugate] concentration of 1/50,000 (Hansen *et al.* 2011).

3.2.7.2 *ELISA Procedure*

On day one, the antigen, recombinant mutated onchocystatin protein was immobilised onto ELISA plates (Maxisorp). The antigen was diluted to 5 µg/ml in fresh coating buffer after optimisation by checkerboard titration. The dissolved antigen was dispensed in 100 µl aliquots into the ELISA plates, which were covered with adhesive plate sealer, incubated at room temperature for one hour and then refrigerated at 4^{o}C overnight (24 hr).

On the second day, the coating buffer was discarded from the ELISA plates and freshly prepared wash buffer was used to remove unbound antigen five times. Each washing comprised of incubating the ELISA plate with wash buffer for five minutes before discarding it and refilling the plate with fresh wash buffer. After the final wash, the ELISA plate was tapped onto absorbent material several times to eliminate all residual liquid. Fresh blocking buffer (200 µl) was added into each well and the plate was covered with adhesive plate sealer, incubated at room temperature for one hour, and then overnight at 4^{o}C.

The serum samples were then prepared for use on the third day by removing any antibodies raised against the GST moiety of recombinant onchocystatin. The process of pre-adsorption whereby sera were incubated with *E-coli* GST lysate [(Promega®, Bio-Rad Laboratories, Hemel Hempstead, UK) containing an irrelevant GST and His-tagged protein] to which soya milk was added for two hours at ambient temperature, followed by incubation at 4^{o}C overnight was performed. Final concentrations were 2 mg/ml (protein) for the *E. coli* lysate, 20% soya milk and 10% serum. The supplied *E-coli* GST lysate was reconstituted by adding 7 ml of wash buffer to the vial, incubated at room temperature for 5 minutes, then mixed by swirling and the final volume corrected to 10.7 ml. This was stored as 2.5 ml aliquots in bijou bottles at -20^{o}C.

On day three, the pre-adsorption sera (1/10 dilution) was further diluted to a final concentration of 1/50 with blocking buffer. Following washing as described above, 100 µl of each control or sample serum was added into assigned wells with coated antigen, while an equivalent volume of blocking buffer was added to the conjugate

and sample blank wells. The samples were incubated at room temperature for two hours prior to washing as before. Monoclonal anti-bovine IgG conjugated to HRP was diluted to 1/50,000, and 100µl was added into each well except for the sample blank. Following addition of blocking buffer to the blank wells, samples were incubated at room temperature for 2 hours and then washed as earlier described.

Each well was loaded with 100 µl of substrate ATBS and incubated at room temperature in the dark for 30 minutes. Optical densities (OD 405 nm) were obtained on an Infinite F50 microplate reader (Tecan, Männedorf, Switzerland) at 10, 20 and 30 minutes during the final incubation. The 10-minute reading gave the best response results which were expressed as "per cent positivity"; *i.e.*, the OD of the test serum divided by the OD of the positive control pool, multiplied by 100.

3.2.8 Immunohistology

To quantify nodule eosinophilia, five nodules fixed in 10% buffered formalin were randomly selected using an online service (www.random.org) from each experimental group. One half of the fixed nodule (cut along the longest diameter with a scalpel blade) was embedded in paraffin, cut into 4 µm sections using a microtome, and mounted onto glass slides. The sectioned nodules were washed with distilled water, deparaffinised in glacial acetic acid (1 in 1,500 dilution) for 30 seconds and washed again with distilled water. Thereafter, they were rehydrated (by passing through descending concentrations of ethanol) before a final rinse (in distilled water) followed by stained with Giemsa (1 in 45 dilutions in distilled water) for one hour. Eosinophils were counted across the longest cross-sectional diameter of nodule on an Axio Imager M2 microscope (Zeiss, Oberkochen, Germany) equipped with Zen

2012 software (Zeiss) at ×400 magnifications. Counts were restricted to a rectangular microscopic view (approximately 0.04mm^2) along a central transect. Only eosinophils identified by bright pink intracellular granules (Langworthy *et al.* 2000; Gilbert *et al.* 2005; Hansen *et al.* 2011) were counted and normalised as mean counts per rectangular view.

3.2.9 Statistical Analysis

Data analysis was carried out in SPSS version 22 (IBM Corporation, Armonk, NY, USA). The parametric variables measured on continuous scale were checked for homogeneity, normality, and outliers. Worm viability was estimated from worm motility on a binary scale and analysed using the Cochran–Mantel–Haenszel test, available under the cross-tab procedure on SPSS with time-point as a layered factor and exact Chi Square statistics to determine fitness. The test statistic of choice for parametric data is repeated measures ANOVA. However, a non-parametric Friedman's two-way ANOVA by ranks and a Kruskal-Wallis one-way ANOVA were used because the data could not be normalised even after log-transformation (see section on data QC). Statistics were accepted as significant when the omnibus P-value was <0.05, and post-hoc pairwise comparisons were calculated using the Dunn-Bonferroni test, with 2-tailed asymptotic adjusted p-values of <0.05 considered significant. Correlation analyses were conducted using the Spearman procedure.

3.3 Results

3.3.1 Sample

Five hundred and thirteen *O. ochengi* worms isolated from 210 onchocercomata were analysed for viability (Table 3.3).

Table 3.3: Summary of worm characteristics

Group[a]	Time-point[b] (Week)	Worm density[c] male	female	Median Age[d] (male female)	motility[e] (male female)	Fecundity[f] (range)	Proportion of viable worms[g] (%) Males (n)	Female (n)	Combined (n)	Overall (N)
CON	0	1.6	1.0	1 2	2 2	1 (0 - 3)	100 (11)	100 (7)	100 (18)	97.5 (119)
	4	2.0	1.0	2 2	2 2	2 (0 - 3)	100 (14)	100 (7)	100 (21)	
	8	2.6	1.0	2 2	2 2	3 (0 - 3)	100 (18)	100 (7)	100 (25)	
	12	1.1	1.0	2 2	2 1	3 (0 - 3)	100 (8)	85.7 (7)	93.3 (15)	
	36	2.6	1.0	2 2	2 2	0 (0 - 2)	100 (18)	100 (7)	100 (25)	
	52	1.1	1.0	3 2	2 1	1 (0 - 3)	100 (8)	71.4 (7)	86.7 (15)	
SLT	0	0.7	1.0	2 2	2 2	2 (0 - 3)	100 (5)	100 (7)	100 (12)	97.7 (87)
	4	1.9	1.0	2 2	2 2	2 (0 - 3)	100 (13)	85.7 (7)	100 (20)	
	8	0.9	1.0	2.5 2	2 2	1 (0 - 3)	100 (6)	100 (7)	100 (13)	
	12	1.3	1.0	2 2	2 1	2 (0 - 3)	100 (9)	100 (7)	100 (16)	
	36	1.0	1.0	2 2	2 1	1 (0 - 3)	100 (7)	85.7 (7)	92.9 (14)	
	52	0.9	1.0	2 3	2 2	1 (0 - 3)	100 (5)	85.7 (7)	91.7 (12)	
ADT[h]	0	1.3	1.0	2 2	2 2	2 (0 - 3)	100 (9)	100 (7)	100 (16)	87.4 [h] (86)
	4	0.6	1.0	2 2	2 2	2 (0 - 3)	100 (4)	100 (7)	100 (11)	
	8	1.9	1.0	2 2	2 2	3 (0 - 3)	100 (13)	85.7 (7)	95 (20)	
	12	1.6	1.0	2 2	2 1	2 (0 - 3)	100 (11)	100 (7)	100 (18)	
	36	0.9	1.0	2.5 2	1.5 1	0 (0 -1)	66.7 (6)	57.1 (7)	61.5 (13)	
	52	0.3	0.9	2 4	2 0	0 (0 - 3)	100 (2)	28.6 (6)	44.4 (8)	
OVC	0	2.0	1.0	2 2	2 2	3 (2 - 3)	100 (14)	100 (7)	100 (21)	100 (107)
	4	2.4	1.1	3 2	2 2	2 (0 - 3)	100 (17)	100 (8)	100 (25)	
	8	1.9	1.0	2 2	2 2	2 (0 - 3)	100 (13)	100 (7)	100 (20)	
	12	0.6	1.0	2 2	2 1	0 (0 - 3)	100 (4)	100 (7)	100 (11)	
	36	1.0	1.0	2 2	2 2	3 (0 - 3)	100 (7)	100 (7)	100 (14)	
	52	1.4	1.0	2 3	2 2	1 (0 - 3)	100 (9)	100 (7)	100 (16)	
OVT	0	1.3	1.0	2 2	2 2	3 (1 - 3)	100 (9)	100 (7)	100 (16)	99.1 (114)
	4	2.1	1.0	2 2	2 2	2 (0 - 3)	100 (15)	100 (7)	100 (22)	
	8	2.3	1.0	2 2	2 2	2 (0 - 3)	100 (16)	100 (7)	100 (23)	
	12	1.9	1.0	2 2	2 1	3 (1 - 3)	100 (13)	100 (7)	100 (20)	
	36	1.1	1.0	2 2	2 1	2 (0 - 3)	100 (8)	71.4 (7)	100 (15)	
	52	1.6	1.0	2 2	2 2	3 (0 - 3)	100 (11)	85.7 (7)	94.4 (18)	
Overall		**1.5**	**1.0**	**2 2**	**2 2**	**2 (0 - 3)**	**99.3 (303)**	**92.3 (210)**	**83.9 (513)**	

[a] CON, Untreated Control; SLT, Sublethal Chemotherapy; ADT, Adulticidal Chemotherapy; OVC, Onchocystatin Vaccine Control; OVT, Onchocystatin Vaccine with antibiotic Therapy.
[b] Time after start of chemotherapy. [c] Average numbers of worms per nodule per time-point.
[d] Age was estimated on a four-point scale: 1, young; 2, middle; 3, old; or 4, dead.
[e] Motility was scored on three-point scale: (0, no movement or dead; 1, reduced movement; or 2, normal movements) recorded after incubation of worms for 30 min at 37°C.
[f] Fecundity of female worms were scored on a four-point scale: 0 (non-gravid); and 1, 2 or 3 for gravid worms containing a third, half, or a full uterine load of microfilariae, respectively.
[g] Worms were considered viable when motility were scores ≥1.
[h] This group displayed a statistically significant difference in total worm viability from the control (Cochran-Mantel-Haenszel test, P = 0.003).

All nodules except two (cow 204 at T4 with two females; and cow 238 at T52, with a resolved nodule) contained a single female worm. Twenty-seven percent of the dissected the 210 nodules had no male worm but 58.9% of the isolated worms were males giving an average male worm per nodule density of 1.5 (Table 3.3).

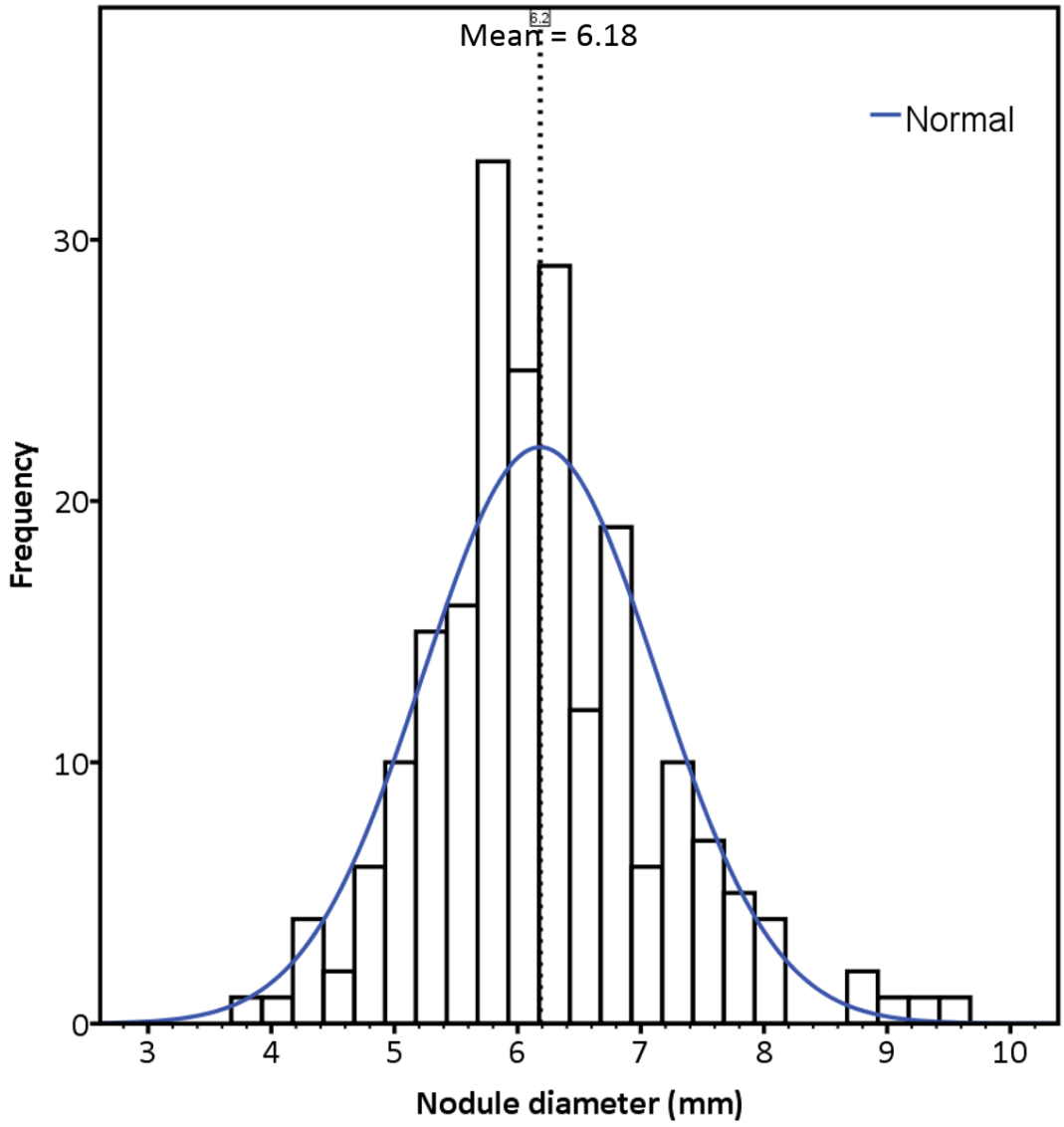


Figure 3.6: Frequency distribution of nodule diameter. Summary data for the entire experiment (N = 210), displaying the observed distribution against a normal curve model generated by SPSS chart builder.

3.3.2 Parasitology

The overall spread of data on nodule diameter was evaluated via frequency distribution, which illustrated an apparent normal distribution (Figure 3.6). The mean nodule diameter (6.2) and the median (6.1) were similar, but slightly higher than the mode of 5.8 (Figure 3.6).

3.3.2.1 *Worm Viability*

Ninety-six percent of the 513 worms studied were alive (Table 3.3). Of the 16 (4%) dead worms obtained, most (14) were females and 62.5% were from the ADT group, including the two dead males recorded at T36. Interestingly, two ADT nodules at T52 (cows 223 and 231) had 4 viable worms (Table 3.3) and the Mf load of the corresponding cows higher than others. One nodule (cow 239) in the control group at T52 had a dead female (Table 3.3) but the present of skin Mf in the cow indicates probable natural worm death from ageing. A Cochran Mantel-Haenszel test statistic was conducted on paired groups comparing treatments to the control taking into consideration time effect. There was an overall statistically highly significant reduction in adult worm viability in the ADT when compared to the untreated control ($P = 0.003$). Dead female worms were also recovered from the SLT (240 at T36 and T52), and OVT (241 at 52) groups (Table 3.3); however, Skin Mf were present and viability in these groups was not significantly different to that in the control.

The ADT therapy significantly affected worm density per nodule site (Freidman's ranked test, $P = 0.017$). However, paired comparisons (post-hoc tests) revealed no statistically significant differences between time-points. Time effect on ADT female worms were more significant (Friedman ranked test, $P = 0.009$) than on the males

(ADT, P = 0.034). Meanwhile, SLT therapy had more significant (Friedman ranked test; P = 0.049) time effect on male worm distribution.

3.3.2.2 *Female Worm Fecundity*

There was no statistically significant difference between group treatment effects on female worm fecundity (Kruskal-Wallis test, P > 0.05). However, in Friedman analysis, ADT therapy statistically significantly (P = 0.036) reduced worm fecundity in the ADT group over time, even though paired comparisons did not reveal significant differences between any paired time points. The lowest median fecundity scores of the ADT group were recorded at T36 and T52 (Table 3.3). Low but insignificant median fecundity was also recorded in the CON and OVC groups at T36 and T12, respectively. There were significant correlations between fecundity scores and the number of viable worms in the CON (Spearman's r = 0.49, P = 0.001), SLT (Spearman's r = 0.49, P = 0.001), OVC (Spearman's r = 0.31, P = 0.48) and ADT (Spearman's r = 0.69, P < 0.001) groups. The fecundity scores of the ADT group correlated positively with nodule diameter (Spearman's r = 0.60, P < 0.001) and the number of male worms (Spearman's r = 0.61, P < 0.001). There was also a weak but significant correlation (Spearman's r = 0.37, P = 0.02) between fecundity and nodule diameter in the OVC group.

Female worms from cows 215 of OVC and 205 of SLT had zero fecundity, even though male worms were present in nodules at most of the time points. Consequently, the Mf load of these animals was zero throughout.

3.3.2.3 *Nodule Diameters*

Changes in diameter of nodules (onchocercomata) is an indirect measure of the effect of treatment on worm viability (Langworthy *et al.* 2000; Bronsvoort *et al.* 2005). Significant reduction in nodule diameters (Friedman ranked test, P = 0.026) was recorded only in the ADT treatment group (Figure 3.7). Nodule diameters tended to decrease as worm viability (Spearman's r = 0.49, P = 0.001) and Mf density (Spearman's r = 0.51, P = 0.001) reduced. Median nodule diameter (4.9 mm, ADT) at T36 was significantly lower than that at T4 (6.4 mm) of the same treatment group (P = 0.009, Friedman ranked test) and that in the OVC group at T36 (5.9 mm; Kruskal Wallis H test, P = 0.035). The SLT therapy had a significant omnibus reduction (P = 0.025) in nodule diameter, but in post-hoc tests, no significant effect was detectable between pairs of time-points.

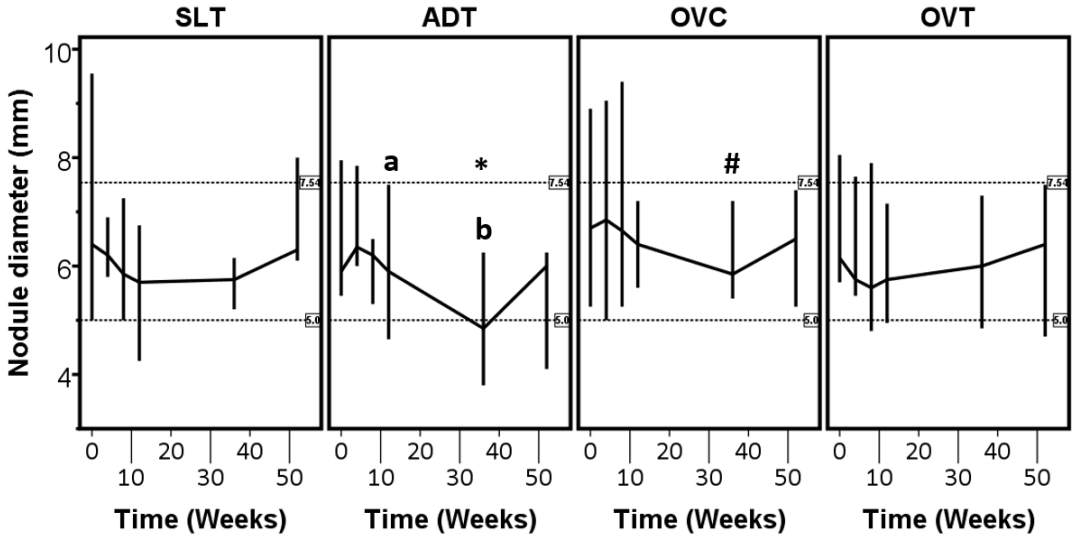


Figure 3.7**:** Effect of immunotherapy and/or antibiotic treatments on *Onchocerca ochengi* nodule diameter. Data are displayed as medians at 95% confidence interval (n = 7). The dotted horizontal lines represent the 5th and 95th percentiles of the untreated control data (n = 42). Points marked by different letters (within a group) or different symbols (between groups) are statistically significant (P = 0.009, Friedman's two-way ANOVA by ranks; P = 0.035, Kruskal–Wallis one-way ANOVA; respectively). SLT, sub-lethal antibiotic therapy; ADT, adulticidal antibiotic therapy; OVC, onchocystatin vaccine control; OVT, onchocystatin vaccine with antibiotic therapy.

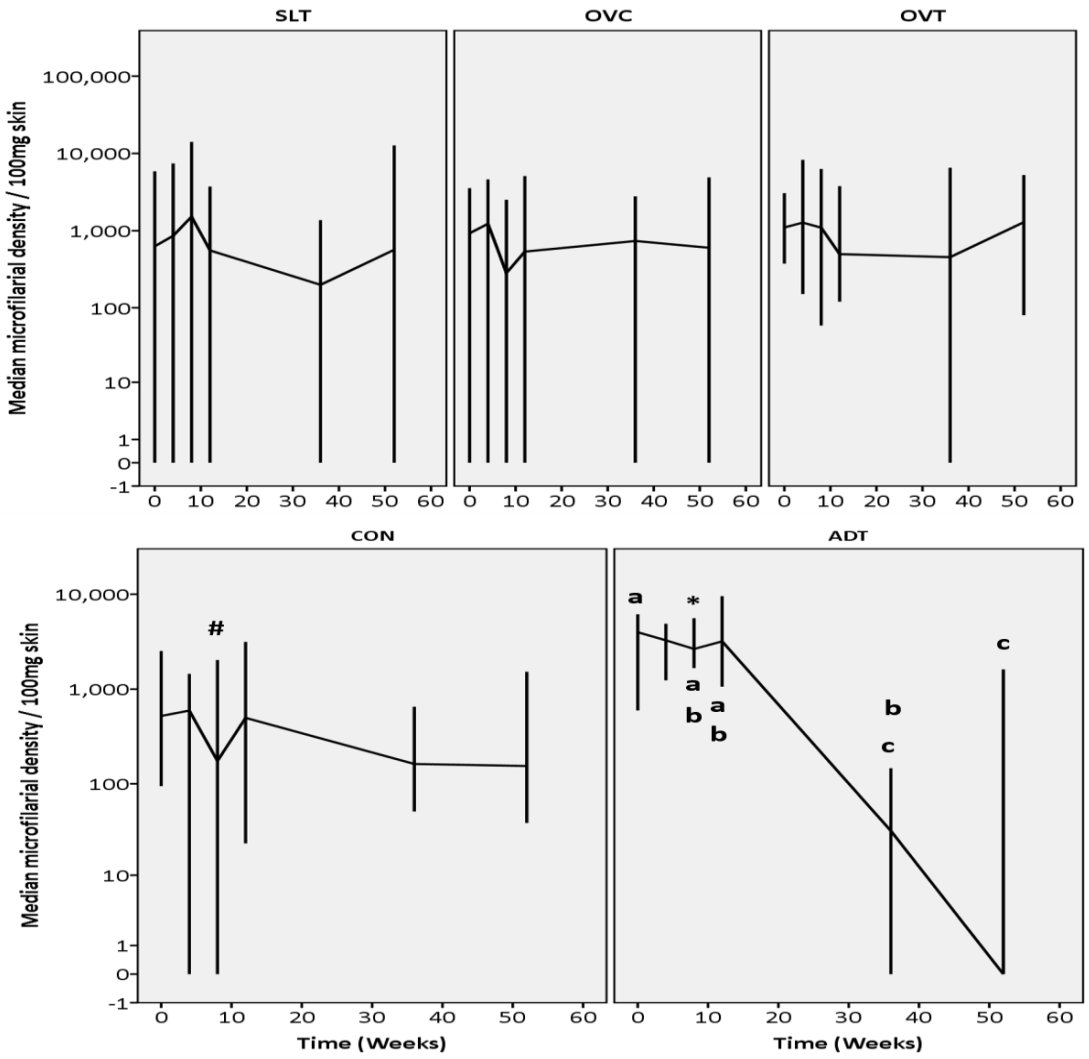


Figure 3.8: Effect of immunotherapy and/or antibiotic treatments on *Onchocerca ochengi* microfilarial density. Medians are displayed at 95% confidence interval (n = 7). Points marked by different letters within a group are statistically significantly different (P < 0.05 for CON, P < 0.001 for ADT; Friedman's two-way ANOVA by ranks), as are points marked by different symbols between groups (P < 0.05; Kruskal–Wallis one-way ANOVA). CON, untreated control; ADT, adulticidal antibiotic therapy; OVC, onchocystatin vaccine control; OVT, onchocystatin vaccine with antibiotic therapy; SLT, sub-lethal antibiotic therapy.

3.3.2.4 *Skin Microfilaria (Mf) Density*

In this study, the group that received ADT had the highest pre-treatment Mf densities (Figure 3.8). The median ADT Mf load was statistically significantly higher (P = 0.027; Kruskal-Wallis H test) than that of the CON group at T8. Though there was no significant between-group difference in Mf densities at T0 and T52 due to the wide

within-group variations, the mean Mf density of the ADT group was about two-fold higher when compared to the other groups at T0, but almost zero at T52 (**Figure 3.8**, ADT). Sub-lethal therapy also statistically significantly reduced skin Mf density (P = 0.036), although pairwise (post-hoc) comparisons were not significant.

The Mf density of the ADT group dropped significantly (P < 0.005) from T0 levels, with median Mf count of > 1000 /100mg skin to zero at T52 (**Figure 3.8**). Notably, four of five ADT animals with dead female worms were negative for skin Mf at T52. Pairwise comparisons within ADT revealed statistically significant differences between T52 and T0 (P = 0.012), T12 (P = 0.015) or T4 (P = 0.032); and between T36 and T0 (P = 0.032) or T12 (P = 0.040), as illustrated in Figure 3.8. Vaccination alone or in combination with antibiotic chemotherapy did not significantly affect Mf load. Interestingly, there were two cattle (cows 205 and 215) of the SLT and OVC groups, respectively, with zero Mf counts throughout the experiment. It was only in the ADT group that Mf density had significant positive correlation with nodule diameter and fecundity (Section 3.3.2.3), and with number of males (Spearman's r = 0.37, P = 0.02) and total viable worms (Spearman's r = 0.49, P = 0.001).

3.3.3 Immunology

3.3.3.1 *IgG Antibody Response to Vaccination*

To determine whether vaccination with onchocystatin generated anti-onchocystatin antibodies, serum specific IgG levels were quantified. As expected, the normalised anti-onchocystatin IgG levels of the CON sera did not change over time (Figure 3.9) and remained approximately equivalent to the positive control pool.

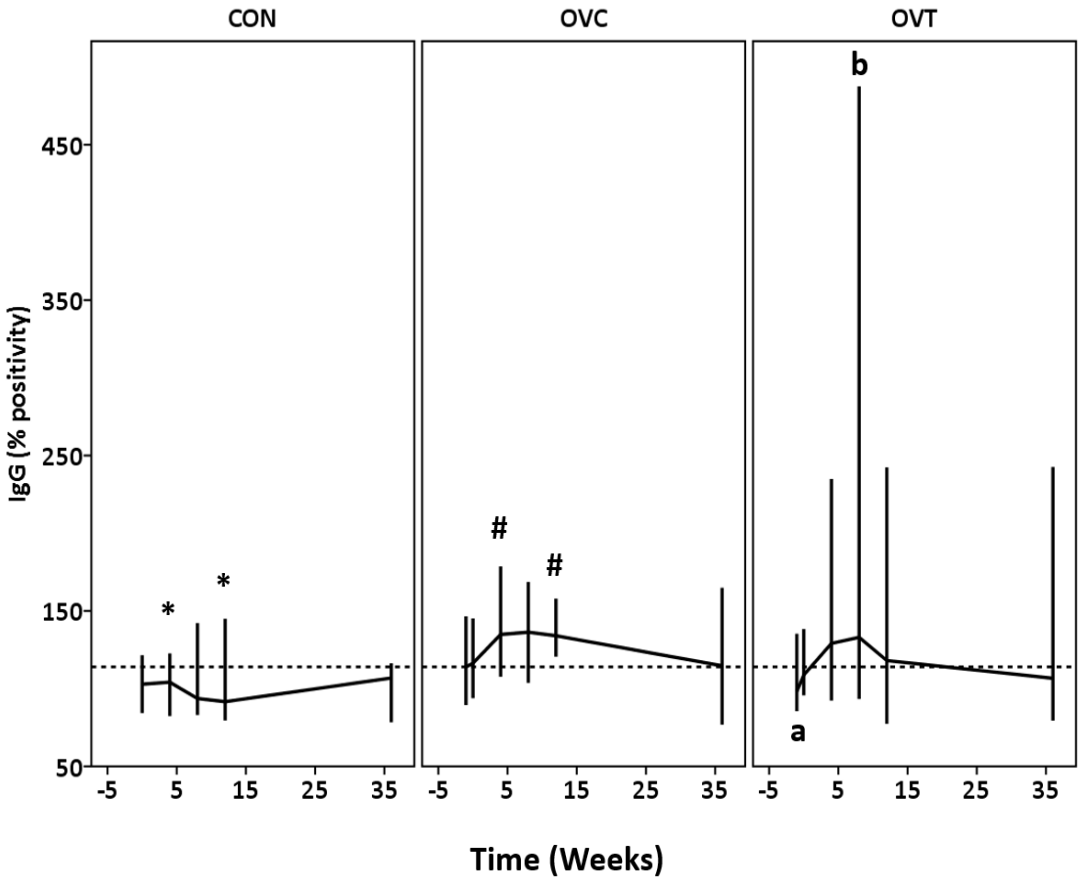


Figure 3.9: Effect of immunotherapy with or without oxytetracycline treatment on anti-onchocystatin antibody levels. Data are displayed as medians (n = 7) and represent per cent positivity relative to a control serum pool (arbitrarily designated 100%) prepared from five untreated, *O. ochengi*-infected cattle from a previous experiment. The horizontal reference line (IgG level, 131%) represents the omnibus median of 126 samples. Points marked by different symbols between groups (P = 0.037; Kruskal–Wallis one-way ANOVA) and those marked by different letters within groups (P = 0.010, Friedman's two-way ANOVA by ranks) are statistically significant. CON, untreated control; OVC, onchocystatin vaccine control; OVT, onchocystatin vaccine with antibiotic therapy.

Vaccination generated a significant response in both the OVC (P = 0.000) and OVT (P = 0.006) groups relative to the CON. Although the omnibus test between the CON, OVC and OVT groups was statistically significant at weeks 5 (P = 0.019), 9 (P = 0.040) and 13 (P = 0.044) post-vaccination (PV), it was only in the OVC group that a post-hoc statistically significant increase in the anti-onchocystatin IgG levels relative to the CON group was apparent at weeks 5 and 13 PV (P = 0.037;Figure 3.9).However, at 9

weeks PV, none of the pairwise post-hoc comparisons exceeded the critical probability. Antibody responses in the OVT group were highly variable, particularly at 9 weeks PV, but significantly higher than the pre-vaccination level (P = 0.010, Friedman's ranked test). One animal from this group (#232) displayed greatly elevated IgG levels throughout the experiment (Figure 3.9). By 37 weeks PV, antibody profiles had returned close to baseline levels in most vaccinated animals.

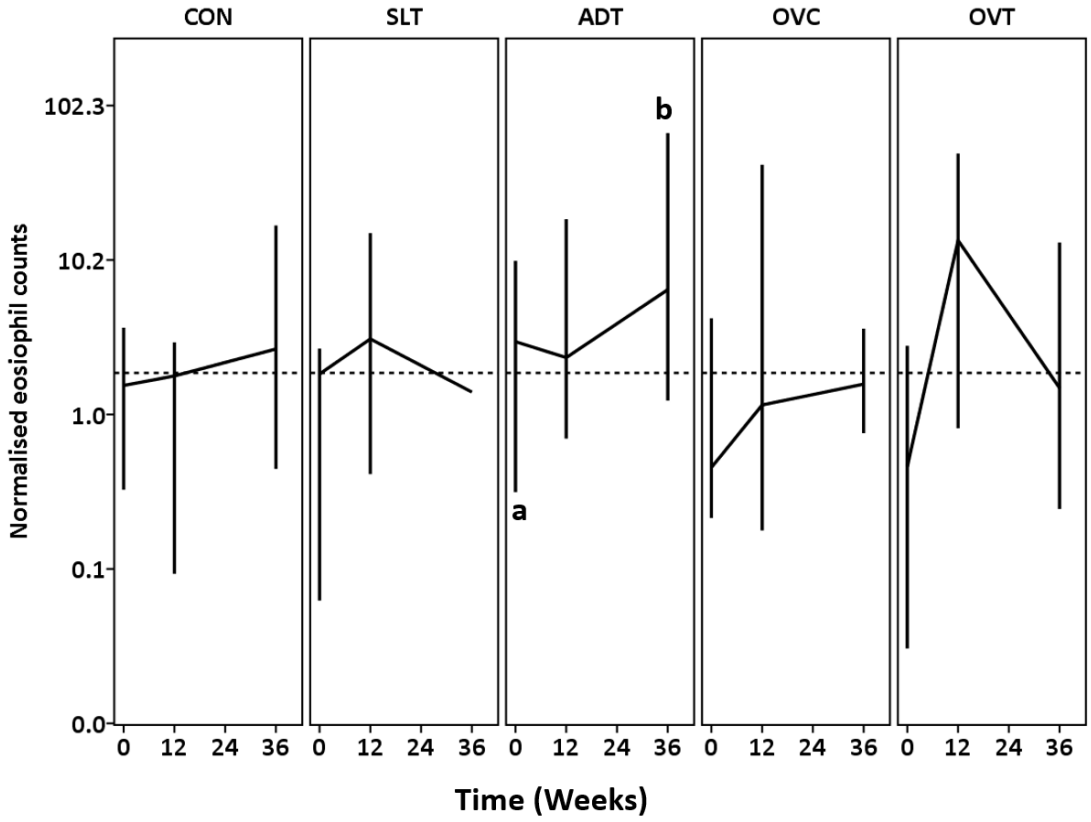


Figure 3.10: Effect of immunotherapy with or without oxytetracycline treatment on eosinophil counts within nodules. The total number of eosinophils counted from all the fields of the longest transect of each onchocercoma was normalised as average count per microscopic view (approximately 0.04 mm^2). The data were plotted as median normalised eosinophil counts (NEC) at 95% confidence interval (n = 5). The broken reference line is the median NEC value of the CON group. The NEC of the adulticidal (ADT) or sub lethal (SLT) antibiotic-treated groups and the vaccinated control (OVC) or immunochemotherapy (OVT) groups were compared to the untreated control (CON) group. Points marked by different letters within a group are statistically significantly different (P = 0.013, Friedman's two-way ANOVA by ranks).

3.3.3.2 *Immuno-histology: Intra-Nodular Eosinophil Infiltration*

To assess whether the increase in serum anti-onchocystatin antibodies resulted in increased intranodular eosinophil load, eosinophil quantification from histological sections of nodules was performed (Figure 3.11). As expected, ADT generated a significant increase (P = 0.013) in eosinophil counts at 36 weeks PV (Figure 3.10) relative to the time zero for this group. Compared to the control, there was no significant increase in the ADT group at T36; nevertheless, the ADT median eosinophil count was 2.4-fold greater than that for CON. The vaccinated groups initially had lower nodule eosinophil counts than both the CON and the ADT groups (Figure 3.10). However, immunochemotherapy generated the highest, albeit insignificant, eosinophil response at T12. Vaccination alone generated a slow increase in intra-nodular eosinophil counts, but the median value at T36 was below that of the CON group (Figure 3.10).

To determine if there were any associations between intra-nodular eosinophil counts and parameters of worm viability, we conducted a correlation analysis and ascertained that it was only in the ADT group that normalised eosinophil counts correlated negatively but significantly with Mf load (Spearman's r = -0.58, P = 0.02). It was difficult to accurately measure the distance of eosinophils to the worm surface in histological sections. The eosinophil counts were categorised into three sub-groups (Figure 3.11) based on the proximity of worm sections (A), the capsule boundary (C), and neither of these features (B) to determine if a treatment attracted more

eosinophils towards the worm surface than did the others. No statistically significant differences were observed in the sub-groups between and within treatment.

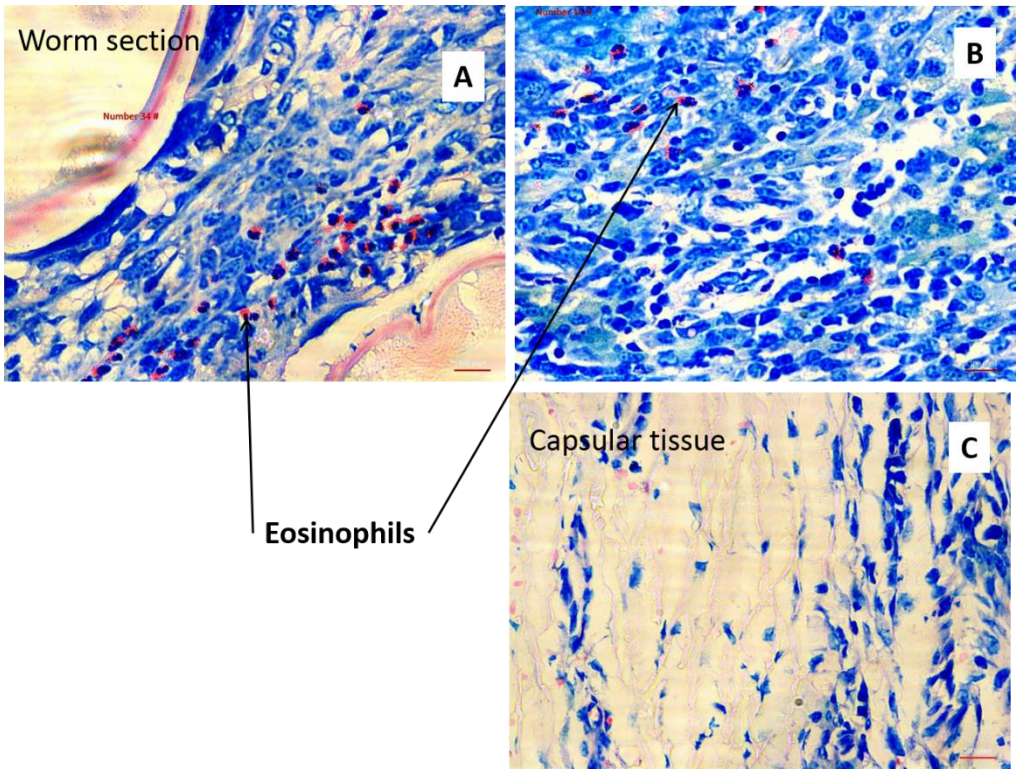


Figure 3.11: Eosinophil count fields regrouped as fields with worm sections (A) or capsular tissue (C), and fields with neither worm sections nor nodule capsular tissue (B). The micrographs were taken with an Axio Imager. M2 microscope (Zeiss) at ×400 from an OVC Giemsa-stained nodule transect at T12. Black arrows indicate eosinophils

3.4 Discussion

This study attempted to answer for the first time whether sublethal antibiotic chemotherapy (SLT) can be enhanced by boosting immune responses targeting onchocystatin, an important immunomodulator. Our data suggest that vaccination before SLT chemotherapy did not recruit sufficient eosinophils to kill the adult worms, even though there was a significant increase in anti-onchocystatin IgG antibody responses post-vaccination. There was a very wide within-group variation in IgG antibody responses, which did not correlate with any of the measures of worm viability. However, median antibody levels were never elevated by more than 40% in the OVC and OVT groups relative to the CON group. Previous studies have shown that sustained depletion of endobacteria *Wolbachia* from all worm tissues, including the ovaries, after antibiotic therapy is responsible for the death of adult *O. ochengi* (Gilbert *et al.* 2005) by reversing the neutrophil/eosinophil balance created by the presence of *Wolbachia* in favour of eosinophils (Hansen *et al.* 2011). However, worm killing occurs most effectively when oxytetracycline is administered over several months (Langworthy *et al.* 2000; Gilbert *et al.* 2005; Hansen *et al.* 2011). The eosinophil is the principal immune cell harbouring the capability to kill large extra-cellular parasites (Delves *et al.* 2011). Eosinophil degranulation occurs when specific antibodies bound to the target cross-link Fc receptors on the cell surface, although activation by interleukin-5 from Th2 cells and other sources is required for optimal activity (Horie *et al.* 1996).

Onchocystatin was used in this study because it is an important potential vaccine candidate for the control of onchocerciasis (Lustigman *et al.* 1992; Gregory and Maizels 2008; Makepeace *et al.* 2009). It is produced by the adult worms and all of the larval stages except mature Mf (Lustigman *et al.* 1991; Manoury *et al.* 2001). Onchocystatin interferes with the normal functioning of antigen presenting cells (APC) and their communication with T-helper lymphocytes, which in turn reduces the production of cytotoxic antibodies from B-cells (Villadangos and Ploegh 2000; Pfaff *et al.* 2002). This tips the balance in favour of regulatory T lymphocytes that activate B-lymphocytes to produce non-complement fixing IgG4 antibodies (Hoerauf *et al.* 2005). As an immunomodulator, onchocystatins are essential for worm longevity and growth (Gregory and Maizels 2008).

The efficacy of onchocystatin vaccines has been tested on several other filarial parasites. In the gerbil model for *Brugia malayi,* the migratory pattern of the adult worms was affected (Arumugam *et al.* 2014b) following immunisation with wild-type *Bm*CPI-2, but when mutated *Bm*CPI-2 was used, significant prophylactic and anti-fecundity effects were achieved (Arumugam *et al.* 2014a). In the mouse-*Litomosoides sigmodonti*s model, the combined use of mutated *Ls*-CPI and *Ls*-ALT in a DNA vaccine approach had significant prophylactic effects especially when adjuvants that boost the Th2 arm of the immune response were used (Babayan *et al.* 2012). In this study, we used alum, a gold-standard adjuvant for stimulation of Th2 immunity in both human and veterinary vaccine production (Lindblad 2004; Mohan *et al.* 2013). This study measured total IgG levels, which in cattle comprises only IgG1, IgG2 and IgG3 subclasses. There were high within-group variations in the response to vaccination,

perhaps due to genetic differences between animals and/or the existing *O. ochengi* parasite load. In addition to onchocystatin, there are multiple active immunomodulatory proteins expressed by filarial nematodes such as galectin-1 (Hewitson *et al.* 2008), ShK toxin domain proteins (Armstrong *et al*. 2014; Armstrong *et al*. 2016), and transforming growth factor-β homologues (Armstrong *et al.* 2016). This redundancy is likely to be a major reason why targeting onchocystatin failed to increase the efficacy of the short antibiotic treatment.

Several independent immunomodulatory mechanisms exist that combine to protect helminth infections and prevent anaphylaxis. Sentinel immune cells such as macrophages produce IL-10 that prevents the development of acquired resistance in schistosomiasis (Wilson *et al.* 2011) and in filarial diseases (Specht *et al.* 2012), while Th2 cells become intrinsically hypo-responsive during chronic infections (van der Werf *et al.* 2013). This state of hypo-responsiveness is not dependent on IL-10 alone (Taylor *et al.* 2005), but represents a lack of ability of $CD4^+$ effector T-cells to proliferate and produce IL-4, IL-5 and IL-2 cytokines (van der Werf *et al.* 2013). Successful immunochemotherapy of chronic filarial infections in the future may require mechanisms that reverse intrinsic Th2 hypo-responsiveness by blocking PD-1 (CD279) receptors (van der Werf *et al.* 2013) and $Foxp3^+$ T-regulatory cells expressing $CD25^+GITR^+$ (Taylor *et al.* 2005).

In vitro studies in mice have shown that immunochemotherapy is achievable in the control of a chronic bacterial diseases (brucellosis) by vaccination with *B. melitensis* outer membrane vesicles at a two-week interval alongside sub-therapeutic doses of doxycycline and gentamicin chemotherapy (Jain-Gupta *et al.* 2014). Indeed,

immunochemotherapy as a strategy has been effective against a range of other pathogens when used as chemically-abbreviated infection or infection-treatment-vaccination against malaria (Peng *et al.* 2014), and in the control of bovine theileriosis (Cox 1992). In cutaneous leishmaniosis, immunochemotherapy with heat-inactivated parasites and sodium stibogluconate favours a Th1 response and rapid healing of skin lesions of patients (Musa *et al.* 2008). In the case of schistosomiasis, there were synergistic effects when sub-curative doses of praziquantel were administered alongside a *Schistosoma mansoni* (*Sm*28GST) DNA vaccine (Dupré *et al.* 1999), a *S. mansoni* tegument nucleosidase vaccine (Rofatto *et al.* 2013), or a recombinant membrane-anchored tegument protein vaccine against *Schistosoma japonicum* (SjTP22.4) in laboratory mice (Zhang *et al.* 2012). In children suffering from schistosomiasis, simple anthelminthic chemotherapy using praziquantel results in accelerated development of acquired immunity (Mutapi *et al.* 1998; Bourke *et al.* 2014). However, there was no post treatment immunisation of *O. ochengi*-infected cattle treated with ivermectin (Njongmeta *et al.* 2004), effective oxytetracycline antibiotic therapy (Nfon *et al.* 2007), and melarsomine (Tchakouté *et al.* 2006). In humans undergoing doxycycline therapy in endemic areas, new infections occurred as worms in the treated nodules were dying (Hoerauf *et al.* 2009).

The antibody (IgG) responses in this study peaked at weeks 5 and 9 post vaccination in the OVC and OVT groups, respectively. Previous immunisation trials used naïve calves to test multivalent sub-unit vaccines (including immunomodulatory proteins, such as unmodified onchocystatin) and attenuated larvae (Tchakouté *et al.* 2006; Makepeace *et al.* 2009). The recombinant vaccine trial elicited significant immune

responses, which peaked at month 4 after two booster doses, ultimately controlling Mf load but not adult worm burden (Makepeace *et al.* 2009). The median antibody production levels in the current study remained at about 40% higher than that of the CON group and peaked at 9 weeks before slightly declining. Subsequent studies should evaluate the effect of one or more booster doses of the vaccine on worm viability; however, induction of tolerance to the antigen is a risk in infected animals (Botros *et al.* 1996).

Although we only measured specific IgG in this experiment, there might have been IgE involvement in ADCC reactions against filariae as in other animal models (Baldwin *et al.* 1993; Abraham *et al.* 2004). However, we measured nodule eosinophil load, which may have interacted with IgE levels (Abraham *et al.* 2004). A significant increase in nodule eosinophilia was observed only in the ADT group, even though the OVT group with lower eosinophil counts at T0 recorded a higher median count than the ADT group at T12. A high within-group variation in the OVT group eosinophil counts was probably responsible for the non-significant immunochemotherapeutic effects observed. The total IgE level in other bovine nematode infections is frequently found to be inversely correlated with worm burden and/or faecal egg counts (Gershwin 2009). In human filariasis, low Mf loads are inversely related to IgG1, IgG2, IgG3 and IgE serum levels (Kurniawan *et al.* 1993), although IgE levels are also strongly associated with post-therapy immunopathology of filarial diseases (Brattig 2004). The interaction of helminths with granulocytes is critically important in host protection, immunopathology and/or parasite establishment (Makepeace *et al.* 2012).

In this study, the anti-onchocystatin antibody response had very low and insignificant correlation coefficients with Mf density, fecundity and number of viable worms. Antibody responses were not evaluated in the ADT group, which had significant changes in all the parameters of worm viability measured. To the best of our knowledge, the role of bovine IgG isotypes in ADCC reactions against filariae has not been investigated, but it is clear that both IgG1 and IgG2 can mediate bovine eosinophil-mediated ADCC to juvenile *Fasciola hepatica in-vitro* (Duffus and Franks 1980).

The recorded reduction in worm viability by the ADT regimen in this study was lower than that (80%) previously reported for this gold standard protocol (Gilbert *et al.* 2005), probably due to the natural adult worm death in the control group. However, our result was similar to that (60%) observed in cattle subjected only to prolonged intermittent antibiotic therapy (Gilbert *et al.* 2005). The current study used twice as many animals per treatment than previous studies on *O. ochengi* (Langworthy *et al.* 2000; Gilbert *et al.* 2005; Nfon *et al.* 2006; Hansen *et al.* 2011), where host mortality among the experimental groups was recorded before the studies were completed. The earliest worm mortality was observed at 8 weeks of ADT from an animal in which dead worms were consistently recovered at later time-points. This pattern was repeated in the four responding animals that had first mortalities at T36. The two cows that responded poorly to the treatment maintained high worm viability at all the time-points, although there was a notable but statistically insignificant decline in their Mf densities at T36 and T52, perhaps indicating some effects on the worms' embryogenesis. In addition, these were the only two animals of the ADT group with

viable male worms at T52. Since our evaluation could not definitively identify dying worms, it could not be concluded whether they were in the process of dying or not. In human studies, significant depletion of the endobacteria *Wolbachia* was observed at 6 months after treatment, and macrofilaricidal effects several months later (Hoerauf *et al.* 2008b; Hoerauf *et al.* 2009). The nodules of the five antibiotic-susceptible cattle that had Mf load of zero were in the process of degeneration, making the identification of males impossible.

The standardisation of the expected number of male worms per nodule site is vital for the evaluation of their viability. Although there were only 2 viable males from 7 nodules in the ADT at T52, 100% male worm viability was recorded because no visible dead male worm could be identified from the degenerated nodules. Previous work have also encountered this problem of male/nodule ratio (Langworthy *et al.* 2000; Gilbert *et al.* 2005; Nfon *et al.* 2006; Hansen *et al.* 2011). Male worms are motile and migrate from one nodule to another. We recorded a maximum of nine males per nodule with an average of 1.5 males per nodule site. Subsequent studies should model the expected number of males per nodule site using data from all the previous studies conducted on the *O. ochengi* model over the last 20 years.

The strong positive association between observed nodule diameter, Mf densities, fecundity, and the number of viable worms observed only in the ADT group indicates that the parameters measured and analysed were true estimates of viability. However, to model the contribution of each measurement of viability, larger datasets will be needed, and parameters monitored for a longer time to ensure total worm mortality in the macrofilaricidal group.

Antibiotic therapy tended to initially precipitate early maturation and release of Mf. ADT treated cattle had a significantly higher Mf load at T8 than did the control group. In previous studies, (Gilbert *et al.* 2005), Mf load tended to increase from T0 to T4 before dropping around T12. The initial response to a stress factor from the adverse environment generated by chemotherapy may be to stimulate embryogenesis or an early maturation and release of Mf before terminating in infertility, sterility (Hoerauf *et al.* 2001; Hoerauf *et al.* 2003; Gilbert *et al.* 2005; Hoerauf *et al.* 2009) and eventual death (Hoerauf *et al.* 2009).

Two cows had no skin Mf at all time-points, though they had at least one male in a nodule with a female worm at all time-points except at T12. Indeed, the worms had good motility and viability scores, but either no intra-uterine Mf were found, or they were in a state of degeneration. Occult filarial infections or amicrofilarial are common in animal models and most human filarial infections (Nanduri and Kazura 1989; Elson *et al.* 1995). These extreme data or outliers were not excluded from statistical analysis and contributed to the wide between group variations observed.

3.5 Conclusions

Immunotherapy using mutated onchocystatin did not enhance the efficacy of sublethal antibiotic chemotherapy against *O. ochengi* because the immune responses generated, and nodule eosinophilia were not sufficiently strong to affect adult worm viability. The positive control antibiotic regimen was adulticidal as expected, and results confirmed the role that eosinophils play in worm killing. The presence of infected cows that appeared to be refractory to oxytetracycline treatment in the ADT group, and an amicrofilaremic cow in the SLT and OVC groups, may warrant further investigations in subsequent studies.

Chapter 4 Proteomics of Oxytetracycline Treated Adult Female *O. ochengi*

4.1 Introduction

Doxycycline, a tetracycline family antibiotic, is recommended for the treatment of individual cases of human onchocerciasis of patients no longer exposed to reinfection and during strategic mass drug administration (MDA) in regions where ivermectin is counter-indicated due to severe adverse effects resulting from co-infection with Loaisis (Hoerauf *et al.* 2008b; Wanji *et al.* 2009). The major limitation of the inclusion of doxycycline in MDA control programs for onchocerciasis is its long therapeutic regimen (Hoerauf *et al.* 2009). In addition, doxycycline is counter-indicated in infants and pregnant women. The mechanism of action of tetracycline antibiotics is associated with its high affinity for 30S ribosomes, leading to inhibition of interactions with aminoacyl-tRNA and thereby impeding protein synthesis (Tritton 1977; Chopra *et al.* 1992). However, the exact mechanism of *Wolbachia* depletion and macrofilaricidal effects of the antibiotic are not known. The elucidation of the mode of action of effective oxytetracycline therapy on *Wolbachia* and anti-*Wolbachia* interactions with the worm will help repurpose tetracyclines. Hence, disrupting *Wolbachia*'s symbiotic relationship with filaria parasites is the focus of much research (Taylor *et al.* 2014). The ability to modify the chemical structure and pharmacokinetic properties of tetracycline (Gurevich *et al.* 1964) led to the production of semi-synthetic derivatives such as doxycycline and minocycline. Predictions made from modelling pharmacokinetic and pharmacodynamics data from rodents indicate that

minocycline can be re-purposed to be more efficacious than doxycycline against *Wolbachia* in lymphatic filariasis caused by *Brugia malayi* (Sharma *et al.* 2016).

In the bovine onchocerciasis, the viability of *O. ochengi* is determined by the type of immune cells surrounding the worm (Hansen *et al.* 2011). Though the pathogenesis related to nodule formation is not clearly understood, it is evident that normal nodules are richly supplied with blood vessels and the worm surface is coated with neutrophils that do not appear to harm the adult worms (Hansen *et al.* 2011). Neutrophils are recruited by macrophages on encountering foreign bodies via cytokines to help immobilise and destroy the invader as a first-line approach to combat the pathogen (Delves *et al.* 2011). Macrophages, as natural scavengers, are typically the first immune cell type to encounter any immunogen or foreign organism that invades a tissue and they also routinely phagocytose dead and abnormal cells. In normal onchocercomata, the filarial endosymbiont *Wolbachia* release peptidoglycan-associated lipoprotein (*w*PAL) whose N-terminal lipopeptide activates TLR-2/6 and cytokine IL-8 to trigger a neutrophilic response (Tamarozzi *et al.* 2016). In chronic conditions or on encounter with extracellular bacteria, neutrophils undergo a form of cell death in which the nuclear DNA and associated basic proteins serve as neutrophil extracellular traps (NET), thereby preventing the spread of infection (Tamarozzi *et al.* 2016). In the bovine onchocercoma, NET formation have not been reported but there is an amorphous, acellular deposit on the worm's cuticle called the Splendore-Hoeppli phenomenon (Nfon *et al.* 2006).

Neutrophils are equipped with three types of granules that contain enzymes and other antimicrobial proteins (Delves *et al.* 2011). The primary azurophillic granules

produce antimicrobial proteins which are either oxidative (myeloperoxidase) or non-oxidative [defensins, bactericidal permeability increasing (BPI) protein and cathepsin G] in nature. The secondary specific granules produce more non-oxidative enzymes such as lysozyme (absent in bovine neutrophils), lactoferrins and alkaline phosphatase than the oxidative enzymes, cytochrome B_{558}. Myeloperoxidase (MPO) plays an important role during the respiratory burst on phagocytosed organisms (Dahlgren and Karlsson 1999). The third type of granules, secreted independent of azurophils, contain two cationic polypeptides (bactenectin and indolicidin) with potent antimicrobial activity (Renato *et al.* 1983; Selsted *et al.* 1992; Selsted *et al.* 1993).

Eosinophils are effector immune cells that induce cytotoxicity, release mediatory cytokines in helminthiasis (Horie *et al.* 1996; Klion and Nutman 2004) and have exceptional capabilities to breakdown extracellular tissues with an arsenal of toxic substances, such as major basic protein (MBP) and eosinophil cationic protein (ECP) stored in a ready-to-use form in granules (Delves *et al.* 2011). Other eosinophil proteins include arylsulphatase B, phospholipase D, histaminase, and preformed cytokines, chemokines and growth factors that, when effectively regulated, exert multiple functions to contain allergy, viral infections, inflammatory disorders and helminthiases, while ensuring minimal damage to surrounding tissues (Klion and Nutman 2004; Delves *et al.* 2011). Eosinophils therefore play additional roles in the maintenance of cell integrity and wound healing, and serve as linkage between the innate and adaptive immune responses (Melo *et al.* 2008; Shamri *et al.* 2011). The release of eosinophils into blood vessels is mediated by IL-5 and / or IL-4 (Pearlman

1997; Pearlman *et al.* 1999) and are activated to confer complement dependent (the preferred site of attachment is on C3b-opsonised parasites) or antibody-dependent (IgG or IgE-opsonised parasites) cell-mediated cytotoxicity (Chandrashekar *et al.* 1990), leading to a piecemeal degranulation (PMD) responsible for regulating the release of cytokines and other proteins during the inflammatory response (Melo *et al.* 2008; Melo and Weller 2010; Delves *et al.* 2011). Eosinophils also exert degranulation by classical exocytosis (Logan *et al.* 2002) and cytolysis following cell lysis to release entire granules within the extracellular space (Melo *et al.* 2008) but PMD is of importance because of the regulatory role it plays in causing inflammatory diseases and in regulation of tissue homeostasis (Melo and Weller 2010). PMD is the main effector mechanism of eosinophils evident by well-structured microtubule subunits within specific granule vesicles or larger sombrero vesicles that permits selective recruitment of cytokines and/or MBP to be transported for release at cytoplasmic membrane (Melo and Weller 2010).

Mast cells are granulocytes found in most tissues of the body especially the skin, respiratory tract and the digestive system. They act as one of primary defence cells and share some functions with macrophages and eosinophils (Urb and Sheppard 2012). The spontaneity of reactions by mast cells are responsible for their frequent association with allergic reactions. Mast cells contribute to parasite killing in much more diverse ways than eosinophils and have been associated with the release of antimicrobials such as cathelicidins (Di Nardo *et al.* 2003) and proteoglycans that regulate cellular activity by induction of apoptosis (Rönnberg *et al.* 2012). However,

previous cytology of bovine onchocercomata showed that mast cells did not contribute significantly to worm killing after antibiotic therapy (Nfon *et al.* 2006).

In contrast with their mammalian hosts, the physiological systems and behaviour of filarial worms are poorly understood (Bain 2002). The inability to grow filarial worms *in-vitro* in culture media throughout their lifecycle has severely limited studies on filarial biology. Intra-nodular *Onchocerca* parasites are in close contact with blood vessels, collagen and connective tissues in addition to the innate immune cells. Their mode of nutrition is not clearly understood, but there is evidence of periodic engulfment of blood (George *et al.* 1985). In the case of *Litomosoides sigmodontis*, worms have been shown to actively ingest blood at early adulthood, shortly after the fourth moult (Attout *et al.* 2005). Female worms of *O. volvulus* or *O. ochengi* are highly entangled and cannot be physically separated from host tissues without inflicting traumatic injuries on the parasite, although enzymatic digestion with collagenase to free the worm is possible.

To better understand the biology of filarial nematodes and particularly the principles underpinning the worm's dependence on endosymbiotic bacteria *Wolbachia* for its survival, there has been rapid development in the sequencing and updating of the genomes of both filariae and other nematode genomes (Laetsch *et al.* 2012; Mavingui *et al.* 2012; Scott *et al.* 2012; Choi *et al.* 2016).

The basis for mutualism between *Wolbachia* of *O. ochengi* (*w*Oo) and its filarial host differs from that of *Wolbachia* of *Brugia malayi* (*w*Bm) in the ability of *w*Bm, but not *w*Oo, to synthesize riboflavin. However, both strains can synthesise haem and might

supply ATP to the worm host (Foster *et al.* 2005; Wu *et al.* 2009; Darby *et al.* 2012). *Wolbachia* cannot synthesise lipid A needed for their own membrane formation and may depend on its host parasite for the supply of other lipids (e.g., cholesterol), as well as amino-acids required for energy metabolism (Elodie Ghedin *et al.* 2007). An estimated 11,508 and 12,143 proteins were predicted from the complete genome assembly of human *Brugia malayi* (95 MB) and *O. volvulus* (97 MB), respectfully (Elodie Ghedin *et al.* 2007; Cotton *et al.* 2016). Computer simulation of metabolic capabilities of *O. volvulus* and that of *L. loa* highlighted mutualism in purine metabolism in *O volvulus* and *Wolbachia* and 16 potential drug targets (Cotton *et al.* 2016). Analysis of the predicted *w*Bm proteins suggested four categories of genes could be targeted to disfavour underlying mutualism between the parasite and its endosymbionts (Holman *et al.* 2009).

The complete proteome of *O. ochengi* was characterised across the major stages of the lifecycle and in nodule fluid recently, providing baseline data (Armstrong *et al.* 2016). The use of proteomics to evaluate post-antibiotic changes in the lymphatic filaria *Brugia malayi* revealed significant changes only in the endosymbiont *Wolbachia* proteins in a study conducted in Mongolian jirds (Dangi *et al.* 2009). In an alternative approach using a mosquito cell line, doxycycline-treated arthropod *Wolbachia* (exposed to the antibiotic for three days only) revealed upregulation of nucleotide and energy metabolism, while outer membrane proteins were downregulated (Darby *et al.* 2014). This provided some insight into the mechanisms used by *Wolbachia* to tolerate antibiotic exposure. However, given the important differences in the genomes between the arthropod and filarial *Wolbachia* strains, it

was necessary to conduct the current *in vivo* long-term, longitudinal study using a natural filarial worm infection.

In the preceding chapter, morphological (worm viability and fecundity, Mf density, nodule diameter and nodule eosinophil counts) and immunological (serology) changes were used to ascertain worm death in response to oxytetracycline antibiotic chemotherapy. This current study is the first analysis of *in vivo* proteomic changes in antibiotic-treated female *Onchocerca* worms. Proteomic changes associated with oxytetracycline-treated *O. ochengi* filarial parasites have never been reported. Given the importance of proteomic technology in systems biology, its use in the characterisation and modelling of cell death mechanisms is progressing rapidly (Chernobrovkin and Zubarev 2016). Advances made in bioinformatics have facilitated the modelling of interactions between proteins, while linking expansive genomic and proteomic datasets to highlight important biological processes (Szklarczyk *et al.* 2015). These analyses are aimed at testing the hypothesis that eosinophils and neutrophils play an important role in the antibiotic-mediated killing of *O. ochengi* filarial worms.

4.2 Materials and Methods

4.2.1 Experimental Animals

Twenty-one cattle, a subset of the 35 Ngaoundere Gudali zebu cattle (Figure 3.1, *Bos indicus*) described in section 3.2.2.1 of this thesis were used for this study (Bah *et al.* 2015). The cattle were aged 6.26 ± 0.46 years by dentition and weighed 278.7 ± 6.9 kg. The ethical considerations, feeding, health care and general management of the animals were reported in section 3.2.2.3.

Cow 233 of the control group aborted during the early phase of the experiment and was administered a procaine penicillin-based antibiotic, Combikel® 40 L.A Kela (20 ml deep intramuscular injection). The cause of the abortion was not determined.

4.2.2 Experimental Design

A randomized block repeated measure experimental design of twenty-one cattle partitioned into three groups of seven cattle each was constituted for this experiment. Two treatments [Sublethal tetracycline therapy (SLT) and Adulticidal therapy (ADT)] were compared to an untreated control at 0, 12 and 36 weeks from the start of therapy (Table 4.1).

The choice of the time-points (T0, T12 and T36) depended on previous findings, indicating important changes leading to the survival or death of adult worms (Gilbert *et al.* 2005). The experiment was designed to closely monitor proteomic changes within female worms following effective therapy (ADT), while comparing them to ineffective chemotherapy (SLT), and the untreated control (CON) undergoing natural ageing or worm death.

Table 4.1: **Experimental block for female worms collected from each treatment group (total *N* = 63).**

Treatment (number of cattle)	Time (Weeks)		
	T0	**T12**	**T36**
ADT (n = 7)	ADT_T0	ADT_T12	ADT_T36
SLT (n = 7)	SLT_T0	SLT_T12	SLT_T36
CON (n = 7)	CON_T0	CON_T12	CON_T36

We hypothesized that neutrophil proteins associated with immune processes which enhanced worm survival and prevented apoptosis of worm cells would be downregulated after antibiotic treatment. Conversely, worm proteins that facilitate resistance to stressors and enhance survival would be expected to be upregulated. Also, eosinophil proteins and processes that are detrimental to the worm should be upregulated. The experimental design permitted statistical analysis to compare changes over time within the ADT group vis-à-vis those of the control and the ineffective therapy.

4.2.3 Parasitology

4.2.3.1 *Sampling Techniques and Parasitological Evaluation of Worms*

See Chapter 3, section 3.2.5.1 and 3.2.6.

4.2.3.2 *Handling and Evaluation of female Worms in the Laboratory*

In the laboratory, the nodules were carefully extracted from the dermal tissues, the worms removed from the nodule, and the female worm examined for motility

(viability) and fecundity. The whole, individual female worms were frozen at -80°C immediately after evaluation in a minimal volume of sterile PBS. Samples were transported in liquid nitrogen from laboratory facilities at Wakwa to Yaoundé and flown on dry ice to Liverpool.

4.2.4 Proteomics

4.2.4.1 *Nematode Worm Solubilisation*

Female worms were thawed on ice and soluble whole body extract (f*Oo*WBE) obtained as previously described (Armstrong *et al.* 2014; Armstrong *et al.* 2016). Each female worm was added to 1 mL of freshly prepared lysis buffer [50 mM Tris, 6 M urea, 1.5 M thiourea and 66 mM dithiothreitol (DTT, Sigma®)], Complete Protease Inhibitor Cocktail (Roche®), a 1:1 mixture of 1 mm glass and 0.1 mm zirconia-silica beads (1 mL) and homogenised for four, 1-min cycles at top speed in a Mini-Beadbeater (Biospec, Bartlesville, OK, USA). Between each cycle, the homogenate was incubated on ice for 2 min. Soluble proteins were precipitated from the supernatant with an equal volume of 20% (w/v) TCA in acetone during incubation on ice for 1 hour, then centrifuged at 12,000 g for 20 min at 4°C. The protein pellet obtained was washed with 1 ml ice-cold acetone and incubated at -20°C for 10 min twice, then rinsed in 200 µl acetone for 10 min at 4°C. After each wash, the proteins were pelleted by centrifugation at 12,000 g and incubated at room temperature for 2 – 5 min to completely dry.

4.2.4.2 *Re-solubilisation of Protein*

The protein pellet was re-suspended in a minimum of 200 µl of re-solubilisation buffer (25 mM ammonium bicarbonate, 6 M urea, 1.5 M thiourea in HPLC grade

water) by repeated flushing of the pellet with buffer using a pipette without vortexing. The re-suspension was completed by sonicating the sample for five minutes (with a between-cycle rest on ice for two minutes) in an ice-water bath over four cycles or more, until the pellet was completely dissolved. The re-suspended f*Oo*WBE solutions were stored at -20°C while setting aside an aliquot (50 µl) for quantification and tryptic digestion.

4.2.4.3 *Protein Quantification*

The concentration of protein obtained from fOoWBE was determined using the Pierce Coomassie Plus (Bradford) Protein Assay (Thermo Scientific). A standard consisting of bovine serum albumin solutions (25, 20, 15, 10, 5 and 2.5 µg /mL) was prepared using the manufacturer's guidelines (Thermo Scientific). Worm protein samples (10 µl) were prepared in triplicate for the Bradford Protein Assay by serial dilutions from an initial 1:100 dilution to 1:200, 1:400 and 1:800. An equal volume (150 µl) of each sample and Coomassie Plus stain was thoroughly mixed, incubated for 10 min at room temperature in the dark, and protein concentrations read at 595 nm on a TECAN® microplate reader. Protein concentrations were determined by linear regression using Magellan software (Tecan).

4.2.4.4 *Tryptic Digestion*

To minimise interference in the tryptic digestion process, the urea molarity of each sample containing 100 µg protein was reduced to at most 1 mMol by dilution in at least 160 µl HPLC water. Proteomic-grade trypsin complex (Sigma®) dissolved in 10 µl of 9 mMol (33 mg/ml) iodoacetamide (Sigma®) and 10 µl of 3 mMol DTT was added to samples at a protein:trypsin ratio of 50:1 and incubated overnight (12 – 16 hours)

at 37°C. Digestion was stopped by adding trifluoroacetic acid (TFA) at 1 µl per 200 µl of sample to reduce the pH. To confirm effective digestion (*i.e*., absence of protein bands), an aliquot (40 µl) of each digested soluble f*Oo*WBE was analysed using SDS-PAGE set at 200 volts and 70 mA for 50 minutes and stained with Coomassie Blue G250 (Ezblue™ Gel stain, Sigma) after heating at 90°C for 5 min. A 5 µl aliquot of Bio-Rad® protein ladder was added to the first and last wells of the gel to serve as an undigested control.

4.2.5 Mass Spectrometry

4.2.5.1 *Desalting and Concentration of Peptides*

The digested peptide samples were spun at 14,000 g for 30 min at 4°C, the supernatants removed by drying in a vacuum evaporator, and the pellet re-suspended in equilibration solution [0.5% TFA in 5% acetonitrile (ACN)]. Sample solution (2% TFA in 20% ACN), wash solution (0.5% TFA in 5% ACN) and elution solution (70% ACN) were prepared. Each sample was reconstituted in 400 µL of equilibration solution. Thermo Scientific Pierce® C18 Spin Column containing reverse-phase resin with minimum (10 ng) and maximum (30 µg) peptide-binding capacity was activated with 200 µL ACN and used to desalt the sample in four steps (Thermo Scientific protocol). The activated C18 Spin Column was wetted with 200 µL of equilibration solution, and the column was spun twice at 1,500 g at each stage and the wash discarded.

During the sample binding phase, the first flow through was re-introduced into the column to maximize peptide binding, centrifuged twice as described above, and the second flow-through discarded. The columns were then washed twice with wash

solution and the peptides eluted into a new receiver tube with 20 µL aliquots of elution buffer. The eluate was vacuum dried, and the purified peptides re-suspended in 3% (v/v) ACN, 0.1 % (v/v) TFA to a concentration of 0.2 µg/µl for analysis by mass spectrometry (MS).

4.2.5.2 *NanoLC MS ESI MS/MS Analysis*

Liquid chromatography (LC)-MS was run by Dr Stuart Armstrong (Armstrong *et al.* 2014). Briefly, the concentrated peptide was analysed by on-line nanoflow LC using the nanoACQUITY-nLC system (Waters) coupled to a linear trap quadrupole (LTQ)-Obitrap Velos (ThermoFisher Scientific, Bremen, Germany) MS equipped with the manufacturer's nanospray ion source. The nanoACQUITY UPLC BEH130 C_{18} analytic column used had a 1.7 µm particle size and 15 cm × 75 µm capillary diameter. The column was maintained at 35°C with a flow-rate of 300 nL/min and the gradient for in-gel digests consisted of 3 - 40% ACN, 0.1% formic acid for 90 min then a ramp of 40 - 85% ACN, 0.1% formic acid for 3 min in positive ionisation mode. Full scan MS spectra (m/z range 300 - 2000) acquired by the Orbitrap at a resolution of 30,000 were analysed in data-dependent mode, and the top 20 most intense ions from MS1 scan (full MS) were selected for tandem MS by collision-induced dissociation (CID). All product spectra acquired in the LTQ ion trap and Orbitrap (with maximal injection times set to 50 ms and 500 ms, respectively) were imported into Progenesis QI (version 4.1, Nonlinear Dynamics, Newcastle, UK) for protein identification and quantification.

4.2.5.3 *Protein Identification and Quantification*

The spectra in Thermo RAW format imported into Progenesis QI were aligned by the software default settings and alignment scores verified. Samples with alignment scores of less than 50% were omitted from the statistical analysis. Peaks with a charge state between +2 and +7 were filtered in, spectral data transformed into Mascot generic format (mgf) with Progenesis LC–MS (version 4.1, Nonlinear Dynamics) and all peptides in the sample identified using the Mascot [version 2.5.1, Matrix Science, P <0.05, <1% false discovery rate (FDR)] search engine. Tandem MS data were searched against gene models from the *Wolbachia* symbiont, *w*Oo (UniProt release 2015_07; 766 protein sequences); the bovine host (*Bos taurus*, UniProt release 2016_08; 24,214 protein sequences); and a general contaminant database *(*GPMDB, cRAP version 2012.01.01; 115 protein sequences). Data were also searched against a revised *Onchocerca* database generated using the ProteoAnnotator pipeline (Ghali *et al.* 2014) as detailed by Armstrong et al. (2016), which contained 13,419 gene models from *O. ochengi* (http://parasite.wormbase.org/Onchocerca_ochengi_prjeb1809/Info/Index) and 572 from *O. volvulus* (Cotton *et al.* 2016). The search parameters were at a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 (LTQ-Orbitrap Velos) while permitting two missed tryptic cleavages. The fixed modification was carbamidomethylation (cysteine) and oxidation (methionine) was set as a variable modification. The results from the Mascot search were validated using the learning algorithm Percolator and the decoy database function embedded within Mascot. The FDR was set at <1% while individual Percolator ion scores of >13 indicated identity or

extensive homology ($P < 0.05$). Mascot search results were imported into Progenesis QI as XML files for calculation of relative protein abundance by the default Hi-3 method (Silva *et al.* 2006). The abundance of each peptide was calculated from its constituent peptide ions and normalised across samples by average intensity using the Normalyzer software package, such that the protein signal was the average abundance of the three top-ranked peptides (Chawade *et al.* 2014).

Table 4.2: Experimental designs for Progenesis statistics.

Test statistic conducted (sample size)	Comparisons	No. of DE proteins	Percentage
1. Overall within groups (n=57)	All treatments and CON grouped within T0, T12 and T36	2	0.4
2. Within ADT treatment (n=15)	Comparison within T0, T12 and T36	24	5.2
3. Between ADT time points (n=17)	Comparison between T0, T12 and T36	57	12.3
4. Between ADT and CON groups (38)	Between ADT and CON	137	29.5
5. Between ADT, SLT and CON (59)	Only 3 groups factored	107	23.1
6. Between ADT, SLT and CON (59)	9 groups linked to time factored	36	7.7
7. T36 between ADT, SLT and CON (18)	T36 between ADT, SLT and CON comparison	101	21.8
Grand Total (actual total no. of DE proteins excluding duplicates)		**464 (203)**	**100**

T time from day 0 (T0) though week 12 (T12) and 36 (T36) after first treatment to ADT (adulticidal oxytetracycline therapy), SLT (sub-lethal oxytetracycline therapy) and the CON (untreated control) cattle. n sample sizes varied between groups and within groups due to elimination of some samples due to quality control at progeneisis.

4.2.6 Bioinformatics and Other Statistical Analysis

The raw abundances of the identified proteins were subjected to several within-group and between-treatment comparisons using Progenesis statistics (Table 4.2). The percentage score of the significant proteins was calculated with respect to the summed number of proteins from all experiments. Duplicates of proteins that were significantly regulated in more than one statistical comparison were removed from

the final tallies. Prior to analysis, two animals (cows 238 and 231) from the ADT group were excluded from all within-group statistics (Table 4.2: Tests 1 & 2) as their samples did not align with the others in Progenesis. However, cow 231 had a low alignment score (33%) only at T12; therefore, in the between-group comparisons, the other time-points (231_T0 and T36) were included (Table 4.2). It should be noted that worm of the sample resisted treatment at T52. Within group statistics (Table 4.2) were equivalent to repeated-measures ANOVA and between groups, one or two-way ANOVA.

Outputs from these analyses were edited to remove proteins with zero or only one unique peptide to reduce the probability of false protein identifications and to increase the accuracy of quantitative comparisons. Where the statistical power of Progenesis analyses was above 80%, proteins with significant differential expression (DE) having a q-value of ≤0.05 and a fold change (FC) of ≥1.5 were classified as upregulated and/or downregulated. To determine from which component of the worm these DE proteins may have originated, they were searched against 94 *O. ochengi* and 498 bovine proteins found in nodule fluid (NF) as reported (Armstrong *et al.* 2016) and also against 343 proteins expressed in the Splendore-Hoeppli phenomena (SHP) (Armstrong, Hetzel and Makepeace, unpublished data). The predicted protein-protein interactions (PPI) and biological enrichment amongst the DE proteins were searched in String (http://string-db.org/). The String output includes Pfam (http://pfam.xfam.org), InterPro and KEGG pathway (Kanehisa *et al.* 2010) statistics for functional protein domain and pathway predictions (Szklarczyk *et al.* 2015). String edge lengths were shortened by reducing the confidence interval (CI)

to increase the likelihood of identifying PPI. WormBase Parasite (http://parasite.wormbase.org/) was also used to locate conserved domain information for *Onchocerca* proteins, including uncharacterised proteins (Howe *et al.* 2016). Dendrograms to illustrate peptides or proteins with similar trends were obtained from PCA correlations of selected protein profiles to facilitate enrichment analysis. The biological processes of highly enriched pathways with significant false discovery rates (FDR, $P < 0.05$) were obtained and summarised based on Pfam and InterPro domain database output.

Progenesis-reviewed protein statistics and experimental results were exported as .csv files for further statistical analyses (Table 4.2). SPSS version 24 statistical package was used to run correlations between raw protein abundance, worm viability and motility index. SPSS Chart Builder was used to plot changes in worm protein weight over time. Heatmaps of regulated proteins were plotted to summarize FC and protein origin using GENE-E freeware (https://software.broadinstitute.org/GENE-E).

4.3 Results

4.3.1 Undigested Worm Protein

The average weight (±SD) of extracted protein from 63 female *O. ochengi* worms was 2.44 ± 1.07 mg (Figure 4.1). One-way ANOVA of within ADT statistics showed that worms had significantly higher extracted protein weight at T0 than at T12 or T36 ($p < 0.01$) and between the groups, SLT mean protein yield was higher than that of ADT ($p = 0.013$) at T36.

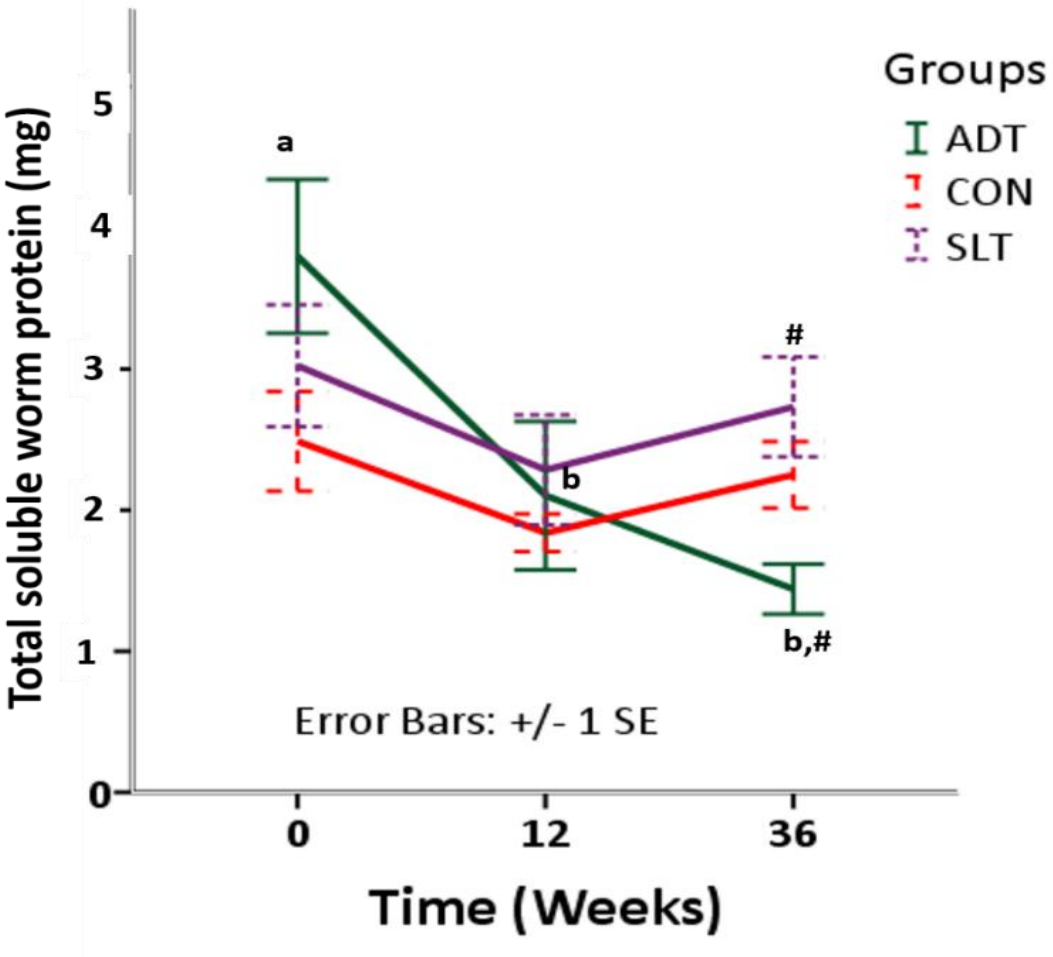


Figure 4.1: Undigested protein profile of female *O. ochengi* worms (N = 63). Key: ADT - Adulticidal oxytetracycline therapy; SLT - Sublethal oxytetracycline therapy; CON - Untreated control; # refers to groups with significant differences ($p < 0.05$); a, b – time-points with significant differences ($p < 0.05$).

Although repeated measure ANOVA (RANOVA) analysis, which is the most appropriate statistical analytical tool for multiple comparisons, yielded no significant differences, we validated the significant results of the one-way ANOVA at T52 based on the fact that female worms of the ADT group degenerated while nodule size significantly dropped at T52. Wider within group variations in extracted soluble protein from ADT worms at T12 compared to those of SLT and CON (Figure 4.1) might have accounted for the insignificant difference obtained from the RANOVA results. These findings suggest that worm proteins abundance could be proxy a measurement for worm viability ($r = 0.31$, $p = 0.017$) as measured by motility.

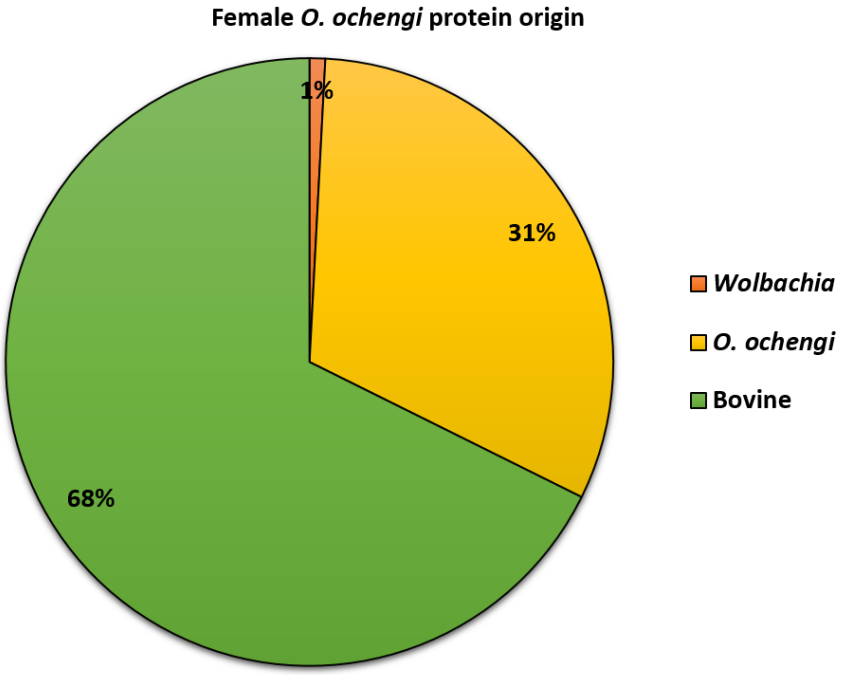


Figure 4.2: Origin of protein extracted from female *O. ochengi* filarial parasite of cattle.

4.3.2 Digested Protein

Two thousand, five hundred and twenty-seven proteins were identified from 12,189 peptides obtained from MS-MS analysis. The identified proteins (Figure 4.2)

originated from cattle (1,583), *O. ochengi* (796) and *Wolbachia* (21). Fifty percent of them retained with two or more unique peptides (Figure 4.3, C) and comprised 35%, 51% and 58% of the respective *Wolbachia*, *O. ochengi* and bovine proteins. Peptide counts were positively skewed (Figure 4.3, A & B).

A. Descriptive Statistics of No. of peptides per Protein identified

	Range		Skewness	
	Min.	Max.	Statistic	Std. Error
Peptide count	1	101	5.880	0.048
Unique peptides	0	77	6.305	0.048
Total No. of Proteins	**2548**			

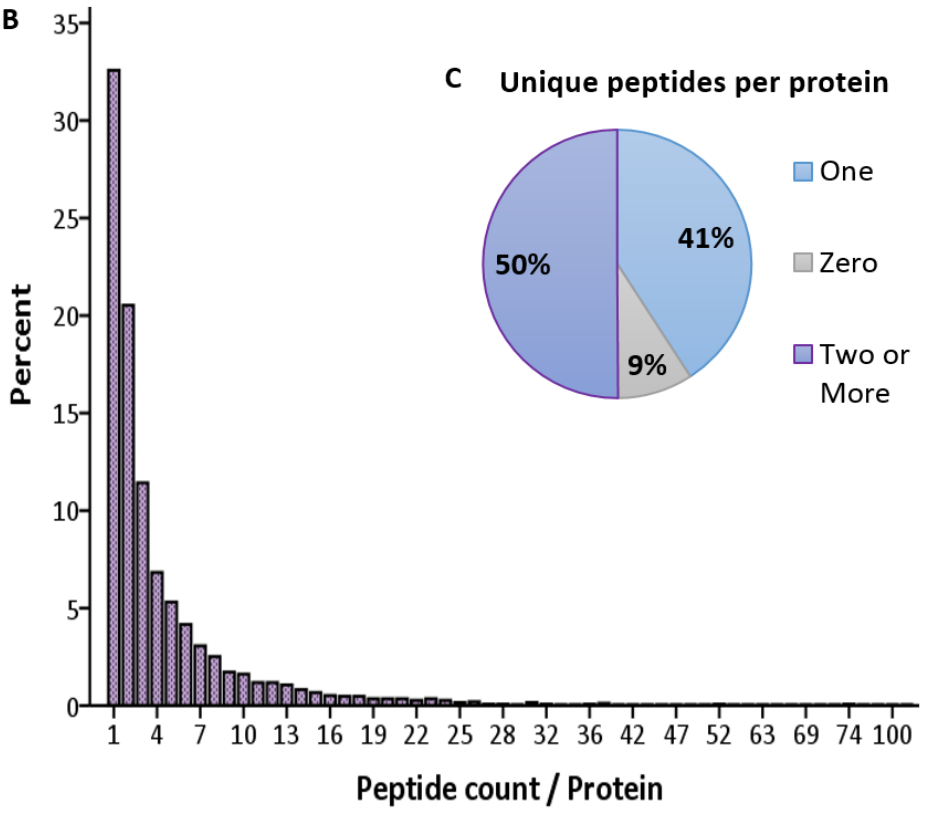


Figure 4.3: Descriptive statistics of (A) peptide count per protein, (B) distribution showing positively skewedness of large sample with unique peptides and (C) proportion (50%) of accepted counts of further Progenesis (n = 2,548).

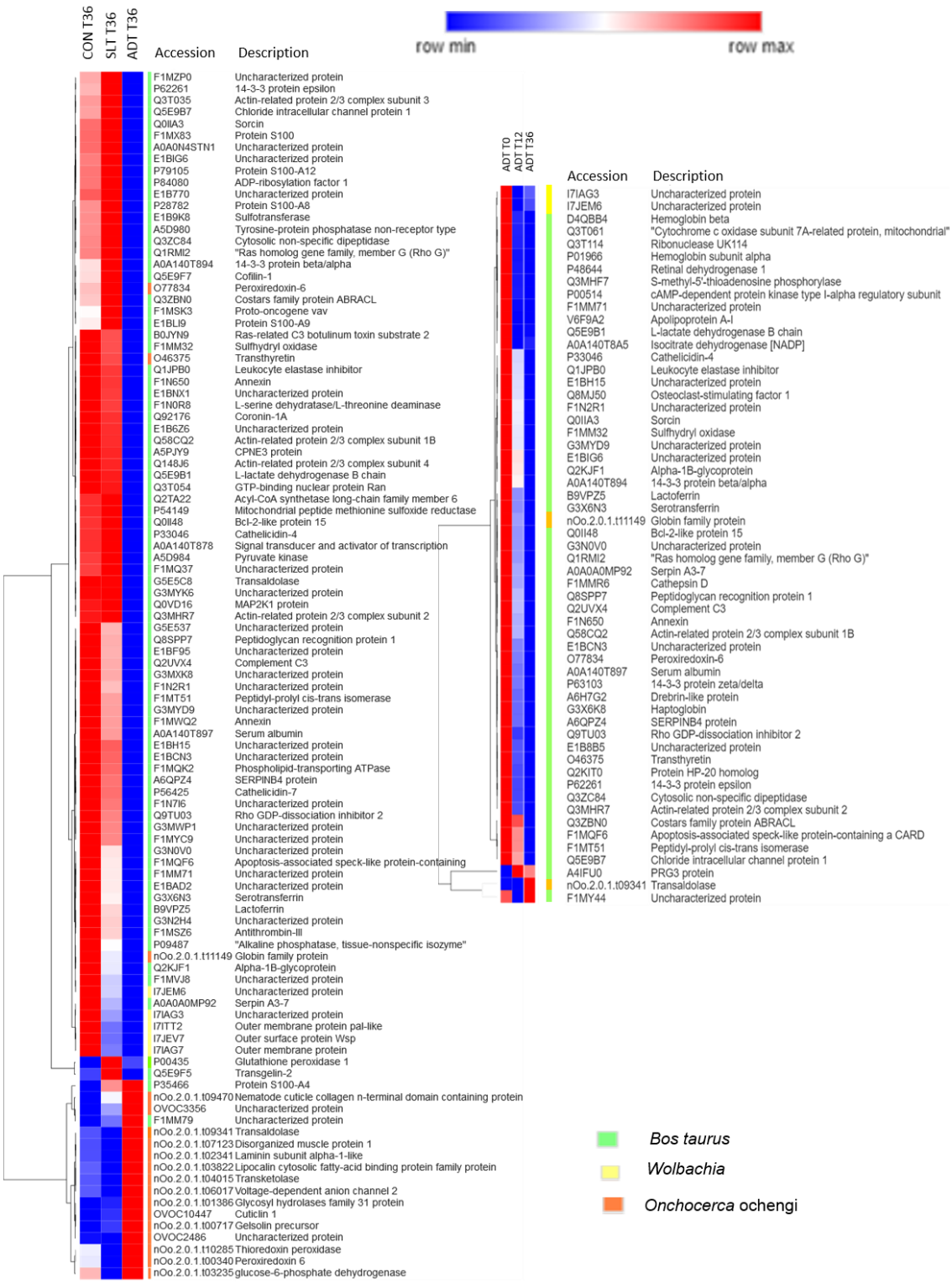


Figure 4.4: Heat map of significant DE proteins (FC ≥ 1.5, q < 0.05) from between groups at T 36 (left) and within ADT (right) test statistics. *Wolbachia* had only downregulated proteins. Unlike in cattle, more worm proteins were upregulated than down regulated.

4.3.2.1 *Regulated Proteins*

Across the seven statistical comparisons performed, a total of 203 proteins exhibited significant DE. The results from two of the seven statistical analyses (Table 4.2) presented (Figure 4.4) accounted for more than two-thirds of the DE proteins, including bovine beta haemoglobin and cytochrome c oxidase sub-unit seven, which were the only two proteins identified by comparison 1 (Table 4.2). An uncharacterised protein with acc. ID G3N0V0 and Rho GDP-dissociation inhibitor 2 were significantly regulated in six of the seven comparisons, and 14 other proteins were significantly regulated in five comparisons. In summary, 124 DE proteins had significant DE in at least two comparisons, thereby inflating the actual number of DE proteins (Figure 4.2). About 40% of downregulated proteins and 50% of upregulated proteins were between the effective ADT and ineffective SLT groups (Figure 4.4).

There were strong correlations between the subgroups of regulated proteins (Figure 4.5, A). While upregulation tended to disorganise protein abundance (Figure 4.5, B), downregulation by ADT lowered protein abundance to the same level (Figure 4.5, C) thereby increasing similarity between them (Figure 4.5, B). Although most of the significant DE proteins occurred at T36, PRG 3, (a homologue of eosinophil major basic protein) had its highest abundance at T12 (Figure 4.4). More than half (119) of the DE proteins been found in nodule fluid (76) and/or SHP (84) and are highlighted in Table 4.3, Table 4.4 and Table 4.5. Forty-one of the NF proteins were also reported in SHP (Armstrong, Hetzel & Makepeace, unpublished).

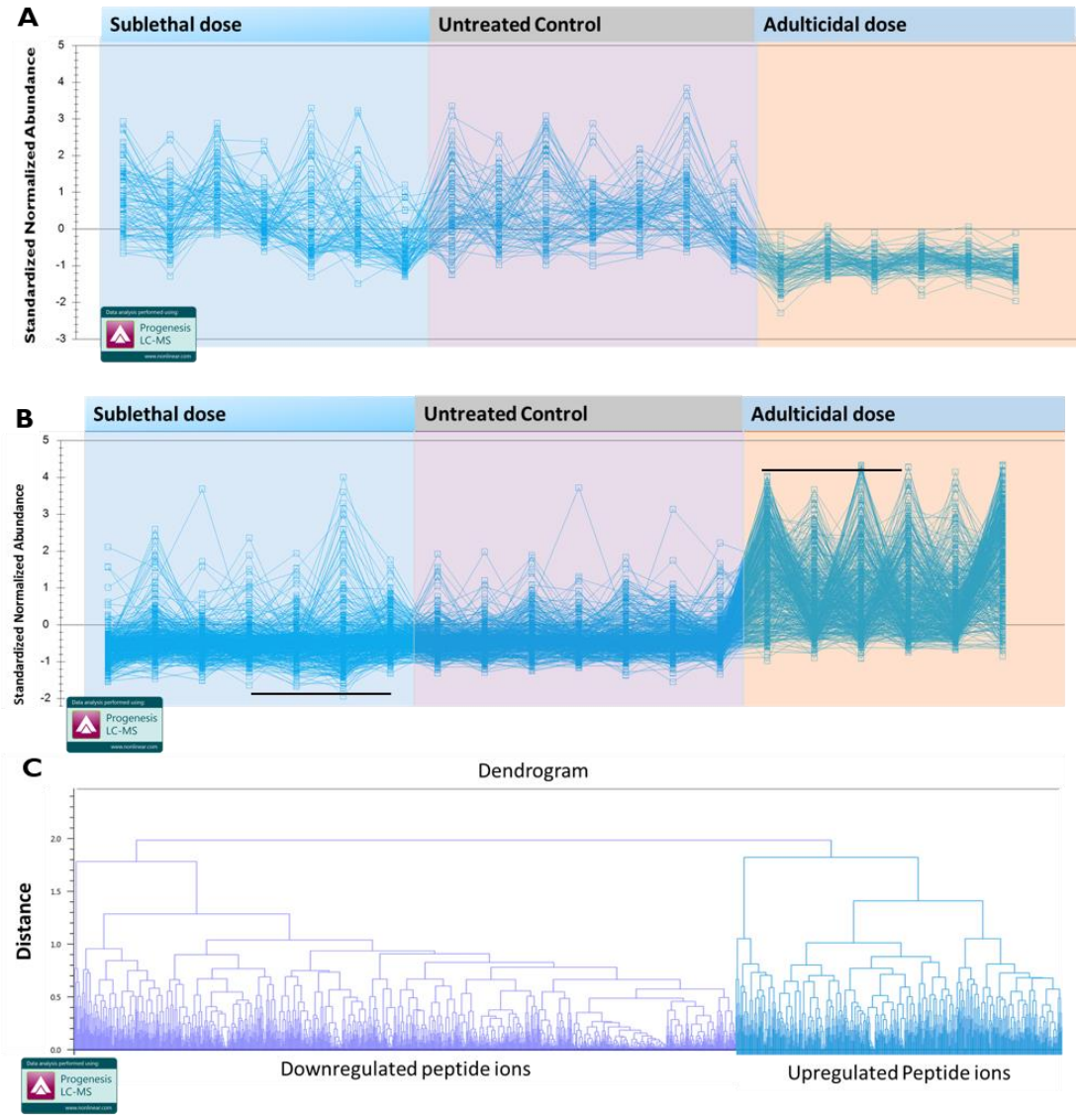


Figure 4.5**:** Between groups normalised peptide ions and protein abundance profiles ($p < 0.002$; FC ≥ 1.5) at T36. Downregulation by ADT (A) tended to normalise protein abundance profiles. The upregulated peptide ions abundance profile (B) illustrates significant differences between ADT and SLT (Dark bar). A Dendrogram of peptide ions illustrating two pedigrees of regulated peptide clusters (C).

4.3.2.1.1 *O. ochengi* Regulated Proteins

Twenty-two female *O. ochengi* proteins had significant DE. Seventeen were upregulated and 5 downregulated (Table 4.3). About half of the *O. ochengi* DE proteins (11) had previously been found in nodule fluid (10) and/or SHP (3) and are highlighted in Table 4.3.

Table 4.3: *Onchocerca ochengi* regulated proteins post oxytetracycline therapy.

Protein Accession	Source	FC-value	q-value	Annotation
		Upregulated		
OVOC10447	iuMf	5.1	0.04	Cuticlin 1
OVOC12310	AF AM iuMf vL3	3.5	0.03	Serine protease inhibitor 1
nOo.2.0.1.t02341[†]	AF AM iuMf NF vL3	3.3	0.05	Laminin subunit alpha-1-like
OVOC3356	AF AM iuMf vL3	2.7	0.04	no description
nOo.2.0.1.t10285 [†]	AF AM iuMf NF vL3	2.6	0.02	Thioredoxin peroxidase
nOo.2.0.1.t09341[†]	AF AM iuMf NF vL3	2.2	0.02	Transaldolase
nOo.2.0.1.t01386[†]	AF AM iuMf NF	2.2	0.02	Glycosyl hydrolases family 31 protein
nOo.2.0.1.t03235[†]	AF AM iuMf NF vL3	2.0	0.05	Glucose-6-phosphate dehydrogenase
nOo.2.0.1.t07123	AF AM iuMf vL3	1.9	0.02	Disorganized muscle protein 1
OVOC2486[†]	AF AM iuMf NF	1.9	0.04	no description
nOo.2.0.1.t04015[†]	AF AM iuMf NF vL3	1.9	0.03	Transketolase
nOo.2.0.1.t09470[#]	AF AM iuMf	1.9	0.03	Nematode cuticle collagen n-terminal domain containing protein
nOo.2.0.1.t00717	AF AM iuMf vL3	1.9	0.05	Gelsolin precursor
nOo.2.0.1.t06017[†]	AF AM iuMf NF vL3	1.6	0.04	Voltage-dependent anion channel 2
nOo.2.0.1.t00340[†#]	AF AM iuMf NF vL3	1.6	0.04	Peroxiredoxin 6
nOo.2.0.1.t03822	AF AM iuMf vL3	1.6	0.2	Lipocalin cytosolic fatty-acid binding protein family protein
nOo.2.0.1.t04870	AF AM iuMf vL3	1.6	0.02	Nematode cuticle collagen domain-containing protein
		Downregulated		
nOo.2.0.1.t00741	AF AM iuMf vL3	1.7	0.01	Ubiquinol-cytochrome c oxidoreductase complex family member (ucr-)
nOo.2.0.1.t04471[†#]	AF AM NF iuMf vL3	4.3	0.01	Beta-galactoside-binding lectin (galectin)
nOo.2.0.1.t11149[†]	AF AM NF iuMf vL3	2.6	0.02	Globin-like protein, involved in oxygen transport
OVOC9820	AF AM iuMf vL3	1.6	0.03	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial
nOo.2.0.1.t08082	AF AM iuMf	5.8	0.03	Dipeptidase C (Aminopeptidase), associated with nematode larval development

Source: represents the different worm stages from which the protein identified previously (Armstrong *et al.* 2016).Keys: AF, adult female; iuMF, intra-uterine microfilaria; AM, adult male. vL3, vector-derived infective larvae; NF, nodule fluid. [#] Protein found in Splendore-Hoeppli deposit and [ł] NF.

4.3.2.1.1.1 Upregulated O. ochengi *Proteins*

Several of the upregulated proteins belonged to the worm's integumentary system comprised of the cuticle, although most were potential excretory-secretory products (constituents of NF proteins), accounting for 53% of the upregulated proteins (Table 4.3). The NF proteins presented identified amongst the regulated worm proteins in Table 4.3 were enzymes involved in redox pathways, the pentose phosphate pathway, glycolysis, and regulation of apoptosis.

An uncharacterised protein with accession OVOC3356 may be a properdin-like protein based on the description of the human homologue (source: UniProtKB; acc: P27918) and has 101 orthologues from 47 other nematodes including *C. elegans*. In mammals, properdins are serine proteases that play an important role in complement activation in cell-mediated immunity and participate in tissue regeneration (Hourcade 2006). Serine protease inhibitors, also known as serpins (Stanley and Stein 2003), were upregulated 3.4-fold ($q < 0.05$) relative to the control group. They are anti-inflammatory proteins essential for the maintenance of haemostasis (Almonte and Sweatt 2011). Gelsolin stimulates the biosynthesis of actin filaments and prevents loss of cytoskeletal monomers in humans (Kwiatkowski *et al.* 1986), while lipocalins transport small fatty acid molecules (Flower *et al.* 1993) associated with inflammation and detoxification processes.

4.3.2.1.1.2 Downregulated O. ochengi *Proteins*

Five worm proteins were downregulated (Table 4.3) at T36 between the ADT and CON groups. One of these, galectin (nOo.2.0.1.t04471), was an NF protein also found in SHP, while globin-like protein (nOo.2.0.1.t11149) was a second downregulated NF protein (Armstrong *et al.* 2016). Globin and two other proteins catalyse processes in the respiratory system, while galectin and dipeptidase C regulate growth. Galectins are abundantly expressed in all tissues both within intracellular and extracellular spaces where they bind to glycosylated proteins and lipids to regulate inflammation and metabolism in humans (Brinchmann *et al.* 2018) and promote survival of parasites by immune modulation and breakdown of blood clots at all developmental stages (Kamhawi *et al.* 2004; Yang *et al.* 2008; González-Miguel *et al.* 2015;

Brinchmann *et al.* 2018). Dipeptidase C (aminopeptidase) was the most downregulated protein (Table 4.3) and functions in the digestion of peptides and oligopeptides. It may be an important source of amino acids for protein synthesis (Taylor 1993).

In summary, changes within worm proteins suggest downregulation of respiration, homeostasis, growth, and metabolism while immune-related environmental stress and antioxidant activities were upregulated.

Table 4.4: Downregulated *Wolbachia* proteins from female *O. ochengi* filarial parasite.

Protein Accession ID	Fold change (q)-values	Protein description	Pfam domain
I7IAG3_9RICK	7.4 (0.00)	Uncharacterized transmembrane	Transmembrane helix
I7ITT2_9RICK	7.2 (0.01)	Outer membrane protein (OmpA)	OmpA-like Integral membrane
I7JEM6_9RICK	4.2 (0.00)	Uncharacterized transmembrane	Transmembrane helix
I7IAG7_9RICK	3.3 (0.03)	Outer membrane (porin 4)	General bacterial porins
I7JEV7_9RICK	2.7 (0.05)	Outer membrane surface antigen	*Wolbachia* surface antigen
I7JET5_9RICK	2.2 (0.02)	ATP-dependent Chaperone protein ClpB	ATPase associated with diverse cellular activities (AAA)
I7JF32_9RICK	1.4 (0.32)	Chaperone protein DnaK (Hsp70)	Heat shock protein 70

4.3.2.2 *Wolbachia Proteins*

Changes in *Wolbachia* protein abundance, being the principal target of oxytetracycline chemotherapy, were expected to exhibit the most significant reduction. A total of seven *Wolbachia* proteins had two or more unique peptides, of which six were significantly downregulated by ADT (Table 4.4; Figure 4.4). These reliably-quantified proteins were identified as the most abundant proteins in *Wolbachia* strain *w*Oo in a previous study (S.L. O'Neill *et al.* 2013). Whereas the within-group protein profile of the ADT treated worms (Figure 4.6, B) illustrated that *Wolbachia* proteins were downregulated at T12 and remained low through T36,

there was no significant change in these proteins from within the SLT (Figure 4.6, C) and CON (Figure 4.6, D) groups.

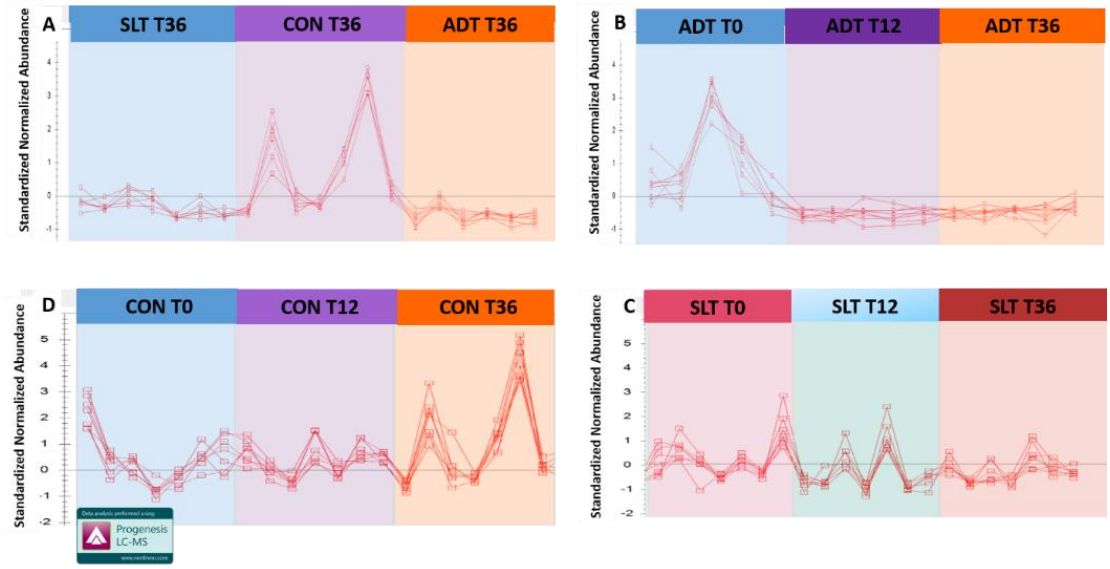


Figure 4.6: Profile of Wolbachia downregulated proteins post-oxytetracycline chemotherapy. A. Comparison between treatment groups at T36. In B, C, and D, protein profiles are displayed for the adulticidal therapy (ADT), sublethal therapy (SLT), and untreated control (CON) groups, respectively.

Transmembrane proteins I7IAG3_9RICK and I7JEM6_9RICK and I7ITT2_9RICK a peptidoglycan-associated lipoprotein (PAL) were the most significantly downregulated *Wolbachia* proteins (WP). The WP with the highest FC (Table 4.4) was a transmembrane protein of molecular weight 25.1 KDa and comprises 237 amino-acids encoded by the wOo_07860 gene (UniProt 2015). Transmembrane helices function as signal transducers and transporters of ions and other substances into the cell by endocytosis (Yin *et al.* 2007). The other two outer membrane proteins and PAL are responsible for neutrophil chemokinesis (Turner *et al.* 2009) and which on activation produce IL8 (Bazzocchi *et al.* 2003). In addition, some WSP form functional complexes with enzymes that are involved in glycolytic pathways (S.L. O'Neill *et al.*

2013) and shown to play important role in the symbiotic relationship with the host with respect to energy dependency (Voronin *et al.* 2016).

Table 4.5: Post antibiotic upregulated bovine proteins included those previously identified in Splendore-Hoeppli deposit and NF.

Accession	Gene code: Protein description	FC (p)-values
A4IFU0	PRG 3: Proteoglycan 3 precursor, calcium dependent apoptosis	5.7 (0.01)
A7MBI5	DPYSL3: Dihydropyrimidinase-related protein 3, nitrogen source	4.0 (0.03)
F1MM79	GLS: Glutaminase; Glutamine catabolism and production of ammonia; nerve cell gaseous exchange	3.9 (0.05)
F1MJU5	BOLA-DRB3: Major histocompatibility complex (MHC) class II precursor, cell mediated immunity	2.9 (0.04)
P35466[†]	S100A4: S100 Calcium/RNA binding protein A4, Modulating proliferation and differentiation of cells	2.9 (0.02)
P13753[†#]	Bota: MHC class I heavy chain isoform 1 precursor; Involved in antigens presentation (By similarity)	2.7 (0.00)
P98133	FBN1: Fibrillin-1 precursor; Microfibrils provide structural support and regulates osteoblast maturation.	2.6 (0.02)
Q2HJA6[#]	NCF4: Neutrophil cytosolic factor 4; Regulatory component of phagocyte NADPH-Oxidase	2.4 (0.03)
F1MY44	HNRNPM: Heterogeneous nuclear ribonucleoprotein M; mRNA processing; fibroblast growth factor	1.8 (0.04)
F1MCK2	Bt.111206: Uncharacterized protein (GN=AHNAK); Voltage-gated calcium channel; poly A RNA binding	1.8 (0.01)
F1MDC1	LOC505941: Endoplasmic reticulum for protein transport; RNA binding; Translation, bone differentiation	1.8 (0.03)
F1MWM8	MRC1: Uncharacterized protein similar to C-type Lectins; Cellular response to IL-4; Mannose binding	1.8 (0.02)
G3N0S8	CYP3A4: Cytochrome P450 family; Oxidation of Xenobiotics; oxidoreductase and heme binding	1.7 (0.01)
P00435[†#]	Glutathione peroxidase 1.	1.7 (0.03)
F1MYG5[#]	LMNA: Prelamin-A/C; Negative regulation of apoptosis and cell proliferation; Response to hypoxia	1.6 (0.02)
P48616[#]	VIM: Vimentin; Regulation of gene expression; glycoprotein and double-stranded RNA binding	1.6 (0.02)
F1MVY8	TAP1: ABC transporter / transmembrane helix; Antigen procession and presentation; ATP binding	1.6 (0.02)
P04272[†]	ANXA2: Annexin A2; Calcium-regulated transmembrane transport; Calcium channel activity	1.6 (0.01)
Q5E9X4	LRRC59: Leucine-rich repeat-containing protein 59; Required for nuclear import of FGF1; RNA-binding	1.5 (0.01)
Q3B8S0	NHP2L1: NHP2-like protein 1; Ribonuclear protein; Plays a role in the late stage of spliceosome assembly.	1.5 (0.01)

[#] Proteins previously found in Splendore-Hoeppli deposit; [†] previously detected in nodule fluid or [#†]both.

4.3.2.3 *Bovine Proteins on Female O. ochengi*

One hundred and sixty-five of the significant DE proteins were of cattle origin. There were fewer upregulated (Table 4.5) than downregulated DE proteins (Table 4.6). The majority (107) of these proteins were similar to those of NF (Armstrong *et al.* 2016) or SHP (Armstrong, Hetzel & Makepeace, unpublished). Thirty-nine of them were common to both NF and SHP.

Table 4.6: Antibiotic Downregulated cattle proteins with significant DE included those previously identified in Splendore-Hoeppli deposit and NF.

Protein ID	Gene code	FC	Protein ID	Gene code	FC	Protein ID	Gene code	FC	Protein ID	Gene code	FC
A2I7M9[†]	SERPINA3-1	38.2	F1MVJ8[#]	OLFM4	3.7	Q5E9F7[#†]	CFL1	2.4	Q3MHP2	RAB11B	1.7
P54229[#†]	CATHL5	8.6	P34955[†]	SERPINA1	3.6	A6H7G2[#†]	DBNL	2.4	F1MDR7	DNAJC13	1.7
Q2KJI3[#†]	FAM49B	7.1	F1MQK2	ATP8B4	3.5	F1MX83[#]	S100A11	2.4	A7MB62[†]	ACTR2	1.7
P09487	ALPL	6.9	O18815[#]	Bt.89877	3.5	F1MM32[#]	QSOX1	2.3	E1BCS3[#]	HK3	1.7
Q8SPP7[#†]	PGLYRP1	6.3	F1MM71[#]	IFIT1	3.4	E1BF95[#]	PPM1F	2.3	E1BH15	CEACAM1	1.7
F1MZP0[#]	GBE1	5.8	F1N7I6	GCA	3.4	F1MZX0[#]	PRKCD	2.2	E1B6Z6[#]	LCN2	1.7
D4QBB4	HBB	5.7	G3N0V0	Bt.97040	3.3	O77834[#†]	PRDX6	2.2	P63048	UBA52	1.7
P33046[#†]	CATHL4	5.6	P79105[†]	S100A12	3.3	F1MWQ2[#]	ANXA3	2.2	E1BNX1	RAB3D	1.7
F6QLM5	GYG1	5.3	G3X6N3	TF	3.2	Q3T114[†]	HRSP12	2.2	F1MCF8	IGLL1	1.7
A0A0A0MP92[†]	Serpin A3-7	5.1	B0JYN9[#†]	RAC2	3.1	Q148J6[†]	ARPC4	2.2	Q3ZC84[#†]	CNDP2	1.7
E1BLI9[#†]	ENSBTAP00000011576	5.0	F1N2W0[†]	PTGR1	3.1	E1BGN3	LOC531990	2.1	Q5E9F5[#†]	TAGLN2	1.7
G3MY87	MGAM	4.9	Q2KIT0[†]	MGC137014	3.1	P84080	ARF1	2.1	Q1RMI2[#]	RHOG	1.7
B9VPZ5[#]	LTF	4.8	Q9TU03[#†]	ARHGDIB	3.1	F1N610	LA-DQB	2.1	Q58CQ2[†]	ARPC1B	1.7
A0A140T897	ENSGT00390000000113.	4.7	Q3ZBN0[#†]	ABRACL	3.0	Q32PF2[#†]	ACLY	2.0	A7Z014	TKT	1.7
P01966[†]	ENSBTAG00000026417	4.5	P14568[†]	ASS1	3.0	A5PJY9[#]	CPNE3	2.0	F1MMK2[#]	G6PD	1.7
Q0II48[#]	BCL2L15	4.5	P48644[†]	ALDH1A1	3.0	F1MYC9	SPTBN1	2.0	A5PKG9[#]	FGR	1.7
G3MXK8	PRTN3	4.4	Q0IIA3	SRI	3.0	F1MS45	CPNE2	2.0	Q0P5A6	PSMD5	1.7
F1N2R1[#]	STOM	4.4	G3X811[#]	ARRB2	2.9	Q3MHF7[†]	MTAP	2.0	P41976	SOD2	1.7
F1MQF6[#†]	PYCARD	4.3	E1BAD2	CTSG	2.8	B1H0W4	NT5C2	2.0	F1MF27	SAMD9	1.7
P19660[#†]	CATHL2	4.1	A5PJH7	NGP	2.8	Q5E9G3[†]	PSME2	2.0	P19120[#†]	HSPA8	1.6
G3X6K8[†]	HP	4.1	A6QPZ4	SERPINB4	2.8	F1MJQ1[†]	RAB7A	2.0	Q3T0K2[#†]	CCT3	1.6
G3N2H4	AZU1	4.0	G3MYD9[#]	ITGAM	2.8	P27214	ANXA11	1.9	F1MHC2[#]	STXBP2	1.6
Q1JPB0[#†]	SERPINB1	4.0	E1B8B5	Bt.66080	2.8	P62261[#†]	YWHAE	1.9	E1BEL7[†]	HSPB1	1.6
E1BIG6[#]	TFRC	4.0	Q92176[#†]	CORO1A	2.8	F1N2F1[#]	FAM129A	1.9	P00514[#†]	PRKAR1A	1.6
P28782[#†]	S100A8	4.0	G3MZG7	LOC783641	2.7	F1MT51[†]	FKBP11	1.9	P32592[#†]	ITGB2	1.6
Q5E9R3[#†]	EHD1	4.0	Q0VD16	MAP2K1	2.7	E1B9K8	SULT1A1	1.9	A5D980[#]	PTPN6	1.6
P56425[#†]	CAMP	3.9	O77774[#†]	NCF1	2.7	A5D984[#]	PKM	1.9	A6QQV8	LBR	1.6
P54149[†]	MSRA	3.9	F1N3U5	VNN2	2.7	Q9GMB8[#†]	SARS	1.9	Q2YDE4[†]	PSMA6	1.6
G3MWP1[#]	ELANE	3.7	Q2TA22	ACSL6	2.6	Q3T035[#†]	ARPC3	1.9	E1BCW3[#]	PFKP	1.6
F1N5K9	ENSBTAG00000010728	3.6	Q3T061	COX7A2L	2.6	G5E5C8	TALDO1	1.9	F1MQ37[#]	MYH9	1.6
V6F9A2[†]	ENSBTAP00000002914	2.8	P13752	Bt.102097	2.6	F1MFD1	PXN	1.8	Q3MHR7[#†]	ARPC2	1.6
G5E537[#]	ENSBTAG00000035224	2.8	Q2KII5[#]	HIST1H2BD	2.5	Q5E9B1[#†]	LDHB	1.8	F1MMC7	MYO1F	1.6
F1MSK3	ENSBTAG00000039160	2.6	F1N650[#]	ANXA1	2.5	F1MWL1[#]	PTPN12	1.8	E1B770	LPCAT2	1.5
G3MYK6	ENSBTAG00000046690	2.4	Q2KJF1[#†]	A1BG	2.5	Q3T054	LOC786258	1.8	F1MFD6	CORO7	1.5
A0A0N4STN1	ENSGT00390000000410	2.3	Q5E9B7[#†]	CLIC1	2.5	F1N6E0[#]	OAS1X	1.8	A5D7Q0	LOC407171	1.5
Q56JW2	ENSBTAG00000009849	2.1	E1BCN3[#]	PADI4	2.5	F1N0Y0[#]	UPP1	1.8	F1MMR6[#†]	CSTD	2.2
A0A140T878	ENSGT00760000119236	2.1	Q2UVX4[#†]	C3	2.5	E1BF48[#]	CD177	1.8			
A0A140T8A5[†]	ENSGT00390000012547	2.0	F1N0R8[#]	SDS	2.5	Q8MJ50[#]	OSTF1	1.8			
A0A140T894[†]	ENSGT00760000119116	1.9	F1MSZ6	SERPINC1	2.5	P63103[#†]	YWHAZ	1.8			
Q3T0E7[#]	ENSBTAG00000040418	1.8	O46375	TTR	2.4	Q3T0V9	DERA	1.8			

[#] Proteins previously found in Splendore-Hoeppli deposit; [†] previously detected in nodule fluid or [#†]both.

Table 4.7: Functional enriched biological processes post antibiotic therapy in female *O. ochengi* worms.

Pathway ID	Description	Obs GC	FDR
GO.0008150	Biological process	64	0.00
GO.0009987	Cellular process	49	0.00
GO.0044699	Single-organism process	49	0.00
GO.0050896	Response to stimulus	35	0.00
GO.0050794	Regulation of cellular process	32	0.01
GO.0051179	Localization	27	0.00
GO.0048518	Positive regulation of biological process	25	0.00
GO.0006810	Transport	25	0.00
GO.0071840	Cellular component organization or biogenesis	24	0.00
GO.0048522	Positive regulation of cellular process	23	0.00
GO.0019222	Regulation of metabolic process	23	0.04
GO.0065008	Regulation of biological quality	22	0.00
GO.0048519	Negative regulation of biological process	20	0.00
GO.0065009	Regulation of molecular function	19	0.00
GO.0006950	Response to stress	19	0.00
GO.0044765	Single-organism transport	19	0.00
GO.0071822	Protein complex subunit organization	17	0.00
GO.0050790	Regulation of catalytic activity	17	0.00
GO.0048523	Negative regulation of cellular process	17	0.03
GO.0002376	Immune system process	16	0.00
GO.0006996	Organelle organization	16	0.02
GO.0009605	Response to external stimulus	16	0.00
GO.0051128	Regulation of cellular component organization	15	0.00
GO.0032879	Regulation of localization	14	0.01
GO.0006952	Defense response	13	0.00
GO.0051130	Positive regulation of cellular component organization	13	0.00
GO.0032268	Regulation of cellular protein metabolic process	13	0.04
GO.0033043	Regulation of organelle organization	12	0.00
GO.0046907	Intracellular transport	11	0.01
GO.0016192	Vesicle-mediated transport	11	0.00
GO.0030036	Actin cytoskeleton organization	10	0.00
GO.0007015	Actin filament organization	10	0.00
GO.0007010	Cytoskeleton organization	10	0.00
GO.0043086	Negative regulation of catalytic activity	10	0.00
GO.0010638	Positive regulation of organelle organization	10	0.00
GO.0015031	Protein transport	10	0.03
GO.0051336	Regulation of hydrolase activity	10	0.01
GO.0006955	Immune response	9	0.01
GO.0051050	Positive regulation of transport	9	0.00
GO.0032956	Regulation of actin cytoskeleton organization	9	0.00
GO.0090066	Regulation of anatomical structure size	9	0.00
GO.0051707	Response to other organism	9	0.00

Keys: ID, identity; obs, observed; GC, Gene count; FDR, False discovery rate. Analysed on String.

STRING analysis (Szklarczyk *et al.* 2015) suggested significant protein-protein interactions (Table 4.8, Figure 4.7), 32 functional enrichments, and 85 biological

processes (Table 4.7) involving 44 cell components. Pfam and Interpro searches identified five and fourteen protein families and domains, respectively, with significant enrichment (Table 4.8).

Table 4.8: Enriched Protein domains and features amongst 166 regulated cattle proteins.

Pathway ID	Description	Obs GC	FDR	Matching proteins in PPI network (labels)
PF00666	Cathelicidin; Polypeptides of lysosome of macrophages and neutrophils	4	0.0007	CAMP, CATHL2, CATHL5, NGP
IPR001894			0.0006	
IPR018216	Cathelicidin, conserved site	3	0.0158	CAMP, CATHL2, CATHL5
PF00071	Ras family; involved cell signal transduction	8	0.0097	ENSBTAG00000010728, ENSBTAG00000046690, LOC786258, RAB11B, RAB3D, RAB7A, RAC2, RHOG
IPR001806	Small GTPase superfamily		0.0108	ARF1, ENSBTAG00000010728, ENSBTAG00000046690, LOC786258, RAB11B, RAB3D, RAB7A, RAC2, RHOG
IPR005225	Small GTP-binding protein domain	9	0.00415	
PF00079	Serpin: Serine protease inhibitor; Irreversible inhibitors and research target.	5	0.0116	Bt.66080, ENSBTAG00000035224, SERPINB1, SERPINB4, SERPINC1
IPR000215				
IPR023796			0.0129	
PF01023	S-100 / ICaBP type calcium binding domain	5	0.001	S100A11, S100A12, S100A8, S100A9, S100A4*
IPR013787	S100/CaBP-9k-type, calcium binding, subdomain		0.0011	
IPR001751	S100/Calbindin-D9k, conserved site		0.0006	
PF00191	Annexin; Ca2+ regulated exocytosis and apoptosis	4	0.0007	ANXA1, ANXA11, ANXA3, ANXA2*
IPR001464				
IPR018502	Annexin repeat		0.0006	
IPR018252	Annexin repeat, conserved site			
IPR002048	EF-hand domain; Calcium binding protein with helix-loop-helix motifs.	9	0.0131	EHD1, GCA, LPCAT2, S100A11, S100A12, S100A8, S100A9, SRI, ANXA2*
IPR011992	EF-hand domain pair		0.0378	
IPR003006	Immunoglobulin/major histocompatibility complex, conserved site	5	0.0211	BOLA-DRB3*, Bota*, Bt.97040, IGLL1, LA-DQB

Keys: ID, identity; obs, observed; GC, Gene count; FDR, False discovery rate. Analysed on String generated Pfam and Intrapro protein enrichements. *Associated chronic conditions.

4.3.2.3.1 Upregulated Proteins of Cattle Origin

Proteoglycan-3 precursor (PGR3) had the highest upregulated FC at T12 (Figure 4.4, Table 4.5). This is the most important finding as PGR3 is an eosinophil major basic protein (MBP) homologue with a C-type lectin-like domain and is a key

helminthotoxic eosinophil granule protein (Specht *et al.* 2006; Gombart 2009; O'Connell *et al.* 2011). PRG-3 is also expressed in mast cells (Rönnberg *et al.* 2012) but in this context, its upregulation is in agreement with previous hypothesis that eosinophil degranulation contributes to worm killing (Nfon *et al.* 2006; Hansen *et al.* 2011). However, this important protein did not appear to have any significant PPI with the others (Table 4.8) probably because sufficient experimental work has not been done to document interactions between bovine granulocytes. The upregulated proteins had significant enrichments (FDR <0.001) in the phagosome (map04145), antigen processing and presentation (map04612), and metabolic pathways (map01100) involving genes (CYP3A4 and GLS) that were specifically associated with microbial metabolism in diverse environment (map01120) alongside downregulated proteins (Table 4.9). Immunoglobin domain (BOLA-DRB3 and Bota) proteins with the conserved InterPro signature IPR003006, calcium binding S100A4 (PF01023), and annexin A2 (PF00191) were among the enriched DE protein families (**Table** 4.8). They contributed to cellular processes regulating transport, organelle organisation and the immune system (Table 4.7) and may be related to increase apoptosis of neutrophils after antibiotic treatment.

Vimentin is an intermediate filament protein and component of the cytoskeleton that facilitates the maintenance of cell shape and cellular integrity (Yue et al. 2016). Along with S100A4, vimentin contributed in many functional enrichments associated with the stabilisation of cytoskeletal interactions (Table 4.7). Protein S100A4 is both a cytoplasmic and nuclear protein and forms complexes with annexin A2 via double calcium binding to regulate several cellular processes during cell proliferation,

migration, and apoptosis (Mirsaeidi et al. 2016). Heterogeneous nuclear ribonucleoprotein M (HNRNPM) is associated with mammalian targets of rifampicin, plays key role in cell survival and growth as well as affect muscle differentiation (Chen *et al.* 2017).

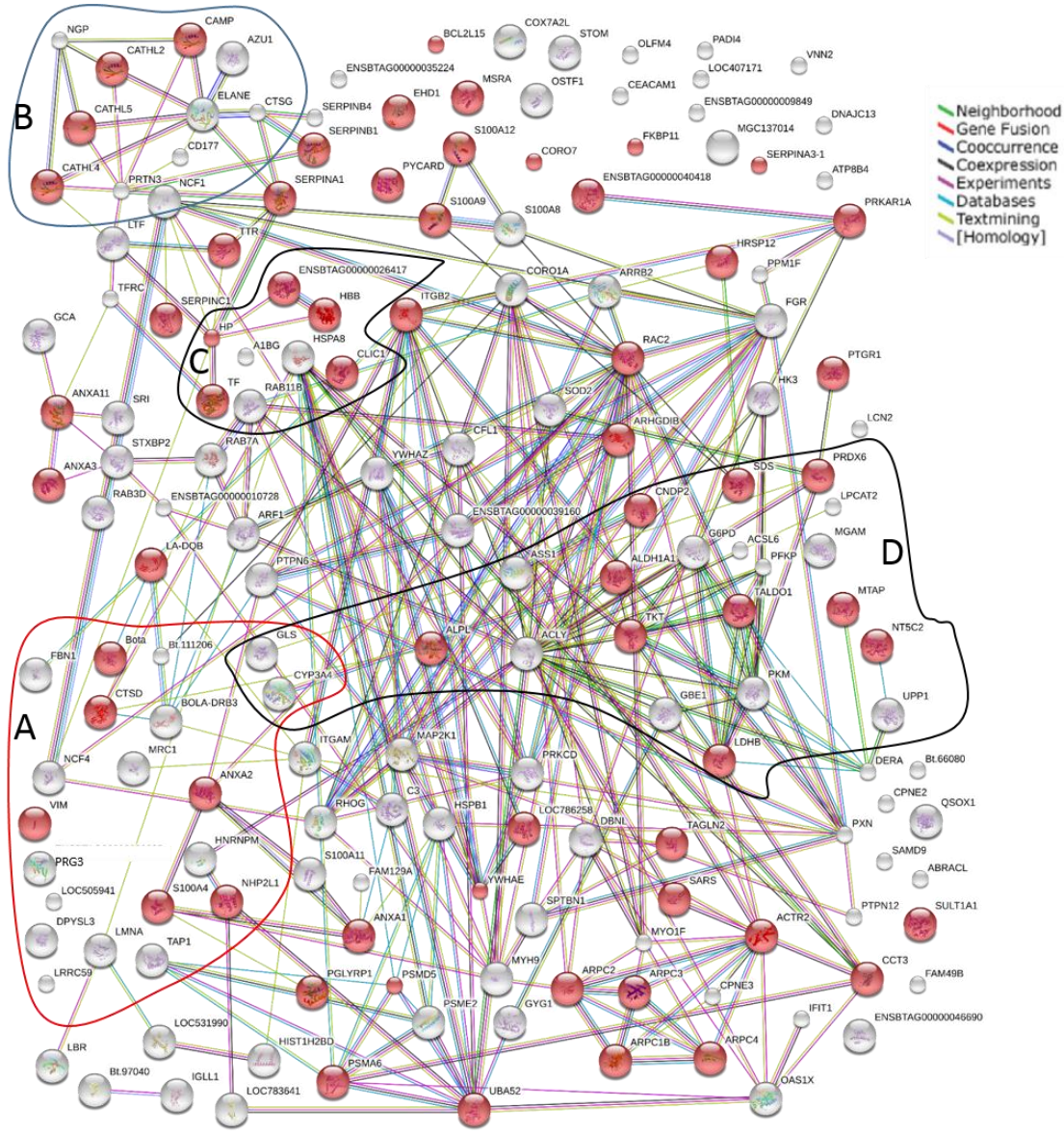


Figure 4.7: STRING network of regulated bovine protein genes in *O. ochengi* filarial worm after oxytetracycline therapy PPI (P = 0.000). Red nodes represent 64 proteins with significant enrichment in biological processes (Go.0008150). Encircled nodes are upregulated proteins (A) CSTD, upregulated between SLT and CON, was downregulated by ADT time-course analysis; neutrophil proteins (B); components of blood (C), and proteins involved in metabolism (D).

Based on curated data containing 122 annotated proteins, HNRNPM and NHP2-like protein 1 had the strongest link via manually-curated metabolic and signalling KEGG pathways (July 2014) for the spliceosome. While HNRNPM and NHP2-like protein 1 are involved in alternative mRNA splicing, processing, and transcript regulatory processes, lamin A/C are key components of the nuclear lamina that regulate nuclear shape and apoptosis. NHP2-like protein 1 is a component of the spliceosome intermediate C Complex (Morimoto and Boerkoel 2013) that contributes in the maturation of mRNA splicing initiated by HNRNPM. The role of these upregulated proteins in this plethora of biological functions that involves majority of downregulated proteins (Table 4.7) will be discussed.

4.3.2.3.2 Downregulated Proteins of Cattle Origin

The downregulated proteins were mostly proteins that surrounded the worm based on high similarity with NF and SHP proteins (Table 4.6). They were proteins of the innate immune response (GO.0002376), specifically neutrophils, with capabilities to both positively and negatively regulate biological processes (Table 4.7). The main enriched protein families and domains were cathelicidins, Ras, serpins and annexins (Table 4.8) that play key roles in Fc gamma receptor-mediated phagocytosis (map04666), leucocyte migration (map04670), and chemokine signalling (map04062), amongst other functions that affect carbon and amino acid biosynthesis (Table 4.9).

Two proteins, haemoglobin beta (D4QBB4_BOVIN/HBB) and cytochrome oxidase (Q3T061|COX7R_BOVIN), were the only proteins with significant DE from the within-group statistics (Table 4.2, Analysis 1). Haemoglobin beta interacts with Haptoglobin

and haemoglobin sub-unit (Figure 4.4, C) which are haemolytic products of the inflammatory response (GO.0045087) and function in the transportation (GO.0006810) of oxygen needed for aerobic respiration, while cytochrome oxidase is the final enzyme in the mitochondrial respiratory chain. These findings may indicate that worms normally contain host blood in the gut, and antibiotic treatment interferes with ingestion of host fluids.

4.3.2.3.3 Pfam and InterPro Protein Domains and Features of Bovine Proteins

Table 4.8 summaries regulated protein domains and features with significant enrichment from amongst the DE proteins on the Pfam and InterPro databases (considered to be well-curated data). Cathelicidin (PF00666), serpin (PF00079), Ras family signal transducers (PF00071) and S-100/ICaBP type calcium binding domain (PF01023) were the protein families identified by Pfam. These proteins also had InterPro conserved signatures (IPR018216; IPR001751; IPR018252; IPR003006) and are involved in cell-mediated immunity (GO.0042742) and modification of the morphology of other organisms (Figure 4.6, GO.0035821), which refers mainly to antibacterial responses. Immunohistochemical evidence from the current study (Chapter 3) revealed a significant reduction of neutrophils at 36 weeks after ADT therapy, suggesting that the cathelicidins were neutrophil proteins (Bah *et al.* 2015). However, macrophages and epithelial cells can also be induced to produce cathelicidins (Di Nardo *et al.* 2003; Liu *et al.* 2006). CATHL-2, -5, -7 (CAMP) and neutrophilic granule protein (NGP) were the four cathelicidins with enrichments (Figure 4.7B). CATHL2 is a potent antimicrobial released during defence response that interferes with the respiratory chain and energy-dependent activities. The other

cathelicidins target both Gram-positive and negative bacteria, while also serving as chemotactic agents and contributing to wound healing (Zanetti 2004).

Table 4.9: KEGG pathways of functional enrichment in antibiotic regulated proteins.

Pathway ID	Description	Obs GC	FDR	Labels of matching proteins in PPI network
04145	Phagosome	13	1.17e-07	BOLA-DRB3, Bota*, C3, CORO1A, ITGAM, ITGB2, LA-DQB, MRC1*, NCF1, NCF4*, RAB7A, TAP1*, TFRC
04666	Fc gamma R-mediated phagocytosis	10	1.9e-07	ARPC1B, ARPC2, ARPC3, ARPC4, CFL1, ENSBTAG00000039160, MAP2K1, NCF1, PRKCD, RAC2
01120	Microbial metabolism in diverse environments	12	3.75e-07	ACLY, ALPL, CYP3A4* ,G6PD, GLS*, LDHB, PFKP, PKM, PRDX6, SULT1A1, TALDO1, TKT
04810	Regulation of actin cytoskeleton	12	5.71e-06	ARPC1B, ARPC2, ARPC3, ARPC4, CFL1, ENSBTAG00000039160, ENSBTAG00000040418, ITGAM, ITGB2, MAP2K1, PXN,RAC2
00030	Pentose phosphate pathway	5	8.61e-05	DERA, G6PD, PFKP, TALDO1, TKT
04612	Antigen processing and presentation	6	0.000558	BOLA-DRB3*, Bota*, HSPA8, LA-DQB, PSME2, TAP1*
01230	Biosynthesis of amino acid	6	0.000653	ASS1, PFKP, PKM, SDS, TALDO1, TKT
05100	Bacterial invasion of epithelial cells	6	0.000761	ARPC1B, ARPC2, ARPC3, ARPC4, PXN, RHOG
04670	Leukocyte transendothelial migration	7	0.00114	ENSBTAG00000039160,ITGAM,ITGB2,NCF1,NCF4,PXN, RAC2
01200	Carbon metabolism	6	0.00313	G6PD, PFKP, PKM, SDS, TALDO1, TKT
01100	Metabolic pathways	22	0.00365	ACLY, ACSL6, ALDH1A1, ALPL, ASS1, CNDP2, CYP3A4*, G6PD, GBE1,GLS*, LDHB, LPCAT2, MGAM, MTAP, NT5C2, PFKP, PKM, PRDX6, SDS, TALDO1, TKT, UPP1
04062	Chemokine signaling pathway	7	0.00822	ARRB2, ENSBTAG00000039160, FGR, MAP2K1, NCF1, PXN, RAC2
04144	Endocytosis	7	0.013	ARRB2, Bota, EHD1, HSPA8, RAB11B, RAB7A, TFRC
04370	VEGF signaling pathway	4	0.0146	HSPB1, MAP2K1, PXN, RAC2
04662	B cell receptor signalling pathway	4	0.0246	ENSBTAG00000039160,ITGB2,MAP2K1,PTPN6,RAC2
00270	Cysteine and methionine metabolism	3	0.0282	LDHB, MTAP, SDS

Keys: ID, identity; obs, observed; GC, Gene count; FDR, False discovery rate. Analysed on String generated KEGG enrichements. *Associated chronic conditions.

NGP is cathelicidin and cysteine-type endopeptidase inhibitor of angiogenesis and lymphoangiogenesis found in cytoplasmic vessicles as well as in the extracellular space, which was co-expressed with CAMP, CATHL2, CATHL5 and CATHL4. CATHL4 is a very potent extracellular microbicide. There were several other uncharacterised bovine neutrophil proteins identified in the network (Figure 4.7, B). Neutrophil

elastase precursor (ELENA) was a primary node that interacted with most of the neutrophil proteins (Figure 4.7). Cathepsin D and AZU1 (uncharacterised chymotrypsin family protein from the azurophil granule lumen) are both serine-type endopeptidases that may be involved in neutrophil-mediated bacteria killing (Zimin *et al.* 2009). In addition, CD177 and PRTN3 (similar to myeloblastin) are neutrophil-derived proteins involved in clot formation and neutrophil degranulation (Figure 4.7).

Beta-defensin C7 (O18815) was another downregulated product that may originate from neutrophil degranulation and is an antibacterial protein which is active in the extracellular space (Selsted *et al.* 1993). It was 3.5-fold downregulated ($p < 0.05$) in the ADT group compared to the control group and is also a component of SHP (Armstrong, Hetzel and Makepeace, unpublished). Neutrophil cytosol (NADPH oxidase) factor-1, NCF-2 and cytochrome P450 are necessary for superoxidase production (Reeves *et al.* 2002). However, cytochrome P450 was upregulated, whereas NCF-1 was downregulated.

RAS proteins regulate cell division and cytoskeletal transport of proteins into the nucleus, and RNA out of the nucleus. Thirty-three proteins linked with myosin and actin cytoskeleton reorganisation (Table 4.9; map04810) were downregulated. KEGG analysis indicated that RAS proteins also function in Fc-gamma-mediated phagocytosis (map04666) and in B-cell receptor signalling (map04662). Downregulated serine protease Inhibitors, annexins and proteins with S100 cacium binding domains are immunomodulators and regulators of apoptosis, reflecting suppression of the neutrophil population at 36 weeks from onset of oxytetracyline therapy.

4.4 Discussion

Proteomics is a branch of high-throughput biology amenable to systems biology analysis (Weston and Hood 2004), and is used to predict functional processes within an organism (Souchelnytskyi 2005). Since the discovery of tetracycline antibiotics as efficacious macrofilaricides for filariae living in symbiosis with *Wolbachia* (Langworthy *et al.* 2000; Gilbert *et al.* 2005), the exact mechanism of adult worm death after depletion of these endobacteria is not known, beyond the understanding that neutrophils are depopulated as eosinophils degranulate on the worm's surface (Hansen *et al.* 2011).

Using proteomic analysis of female worms subjected to adulticidal oxytetracycline chemotherapy (ADT) with 60% efficacy at 52 weeks (Bah *et al.* 2015), we identified proteins from the worm, *Wolbachia* and cattle that are vital for worm survival. We also demonstrated for the first time that the ADT regimen reduced worm protein production in a similar way to Mf load and nodule diameter, until the point where worms were resolved by 52 weeks after treatment. Although tetracyclines interfere with bacterial protein synthesis by preventing amino-acyl tRNA from attaching to the 30S ribosome (Tritton 1977; Chopra and Roberts 2001), the changes in proteins observed were due largely to the downregulation of proteins originating from cattle. In its intranodular lifestyle, female filarial parasites are highly entrapped in collagenous tissues, while the worm's cuticle is coated with SHP deposits (Nfon *et al.* 2006). The contribution of this attached bovine material to worm mass has never been evaluated for *O. ochengi* previously, but the results of this study tend to

illustrate that cattle proteins in close apposition to the worm could constitute more than two-thirds of total protein content. In a stage-specific proteomic analysis of *O. ochengi* (Armstrong *et al.* 2016), collagenase digestion of the nodule reduced bovine protein identifications from 70% to 30% implying that part of the bovine proteins might be ingested alongside host fluid during feeding and are within the worm.

In the current study, more than half of the downregulated proteins were either proteins previosuly detected in SHP, NF or both. SHP is an eosinophilic-stained deposit and a probable product of neutrophil degranulation on the worm cuticle (Nfon *et al.* 2006). The NF proteins constitute an important source for the identification of excretory-secretory products that could serve as biomarkers for the diagnosis of onchocerciasis (Armstrong *et al.* 2016). Our observation is that 60% of regulated proteins present in NF were also detected in SHP, which suggests that many soluble proteins in the the nodular environment are also found in insoluble complexes. Moreover, neutrophil extracellular traps (NET) could be a major source of NF and SHP proteins. NET formation was recently reported in human onchocercomata (Tamarozzi *et al.* 2016). It is probable that NET formation also occurs in the cattle *O. ochengi* model. However, further studies will be needed to test this hypothesis.

STRING analysis of the regulated cattle proteins found in solubilised female worms indicated that proteins of the immune system having significant functional enrichment in Fc-gamma mediated phargocytosis were all downregulated, but 25% of phagosome proteins were upregulated. The abundant phagocytic immune cell surrounding the female filarial worm in its intranodular niche is the neutrophil

(Hansen *et al.* 2011). The upregulation of phagosome proteins may reflect a change in neutrophil activity to digest material released by dying worms. At the onset of infection, at least three *Wolbachia* proteins (GroEL, PAL and WSP) attract and stimulate neutrophils to initiate protective inflammatory response in favour of the worm (Hansen *et al.* 2011). Moreover, WSP inhibits apoptosis in neutrophils (Bazzocchi *et al.* 2007). The downregulation of the most abundant *Wolbachia* proteins (Darby *et al.* 2012) by week 12 preceded those of some neutrophil proteins by week 36 after the start of adulticidal chemotherapeutic treatment and worm death (Gilbert *et al.* 2005; Nfon *et al.* 2006; Hansen *et al.* 2011). Previous immunohistochemistry of ADT-treated onchocercoma reported complete depletion of the *Wolbachia* by eight weeks after treatment start (Gilbert *et al.* 2005). This failure to detect persisting bacteria was probably due to a drop in protein abundance to a level below the threshold for detection by the staining technique. Therefore, the complete depletion of *Wolbachia* was not essential to trigger neutrophil apoptosis and nor was a radical depopulation of neutrophils a precondition for worm death.

The only *Wolbachia* protein for which the abundance was not significantly reduced by ADT was chaperone protein Dnak (I7JF32_9RICK). The role of this protein in *Wolbachia's* ability to resist antibiotic therapy will have to be assessed in future trials before evaluating its chemotherapic potential as target for new drug development. The most downregulated *Wolbachia* protein was a member of a transmembrane protein family (I7IAG3_9RICK). It weighs 25.1 KDa and comprises 237 amino-acids encoded by the wOo_07860 gene (Consortium 2015). Transmembrane helices function as signal transducers and transporters of ions and other substances into the

cell by endocytosis (Yin *et al.* 2007). The other three downregulated *Wolbachia* proteins included those known to act as chemoattractants for neutrophils. In *in-vitro* studies on the effects of doxycycline on *Wolbachia* in a mosquito cell line using directional RNA-seq and label-free quantitative proteomics, membrane proteins were also downregulated (Darby *et al.* 2014).

Energy supply is one of the basis for mutualism between *Wolbachia* and its worm host (Voronin *et al.* 2016). Worm proteins involved in energy regulation such as transaldolase, transketolase, glycosyl hydrolases (Henrissat 1991; Song *et al.* 2013) and glucose-6-phosphate dehydrogenase were upregulated. They catalyse reversible reactions in the pentose-phosphate pathway (Voronin *et al.* 2016) and provide substrates for the tricarboxylic acid cycle (Lindqvist *et al.* 1992; Thorell *et al.* 2000). The endosymbiotic intracelluar α-proteobacteria lack key enzymes needed to convert glucose into pyruvate and therefore, depend on the host filarial parasite for energy (Melnikow *et al.* 2011; S.L. O'Neill *et al.* 2013). In *Brugia malayi*, two *Wolbachia* surface proteins *w*Bm0432 and *w*Bm0152 may interact with worm vacuoles to form functional complexes with enzymes of the glycolytic pathway and cytoskeletal proteins (S.L. O'Neill *et al.* 2013). *Wolbachia* also depends on the catabolism of amino acid from its worm host for energy (Foster *et al.* 2005; Darby *et al.* 2012). Dipeptidase C (aminopeptidase), the most downregulated worm protein after ADT, functions in the digestion of peptides and oligopeptides and provides amino acids for protein synthesis (Taylor 1993). It was first characterised in *Drosophilia melanogaster* and is essential for larval growth, development and locomotion (Hiraizumi *et al.* 1992; Howe *et al.* 2016). The gene encoding dipeptidase C has 189 orthologues, distributed across

53 nematodes and 20 platyhelminth parasites (Howe *et al.* 2016) and is an important target for evolutionary change in helminths.

We were unable to analyse the PPI between species to determine whether the regulated worm proteins interacted with those of *Wolbachia*. However, two worm mitochondrial proteins (ubiquinone cytochrome C oxidoreductase and succinate dehydrogenase iron sulphur sub-unit) were among the downregulated worm proteins alongside a globin-like protein involved in oxygen transportation. A number of the regulated proteins including globin-like protein (nOo2.0.1.t11149) and galectin, a beta-galactoside binding protein have been previously described in NF (Armstrong *et al.* 2016). Several galectins are produced in large quantities by all filarial lifecycle stages (Armstrong *et al.* 2014) and helminth parasites to serve as immune modulators (Dzik 2006; Brinchmann *et al.* 2018), and may disrupt the host's coagulation pathway (González-Miguel *et al.* 2015). In nematodes, globin-like proteins have low affinity for CO and instantly dissociate to the ferric form in the presence of oxygen. Thus, they cannot reversibly bind to oxygen but may act as an oxygen sensor with anti-oxidant properties against reactive oxygen species (ROS) within the neurons of *C. elegans*, rather than as an oxygen transporter (Geuens *et al.* 2010). Different forms of globins regulated by different genes coexist within nematodes (Vinogradov *et al.* 2006). The mechanism of transportation of oxygen in worms is poorly understood. Haem binds with globins to form a complex that transports oxygen from areas of high to low concentration. However, nematodes do not produce their own haem (Foster *et al.* 2005; Luck *et al.* 2016) but are capable of importing exogenous haem (Luck *et al.* 2016), which may be absorbed through the

cuticle alongside other nutrients and oxygen from cattle (Hoogewijs *et al.* 2004; Geuens *et al.* 2010). Previously, the supply of haem to the worm by the endosymbiont *Wolbachia,* particularly during reproduction and larval development in the vector when there is a high demand for energy, was thought to be the bases for worm-bacteria mutualism (Wu *et al.* 2009). However, many filarial nematode species thrive without symbiosis with *Wolbachia*. The dependence on external haem for survival of filarial worms could be targeted to hasten worm death, particular during antibiotic chemotherapy (Luck *et al.* 2016).

Some of the regulated proteins we identified were involved in biological processes that activate or prevent apoptosis within worms. They included ubiquinol cytochrome c oxidoreductases and succinate dehydrogenase. These are mitochondrial proteins (Matsuno-Yagi and Hatefi 1996) that regulate energy metabolism during oxidative phosphorylation and electron transfer (Rao *et al.* 2012). Cytochrome c is a haem-containing protein that blocks mitochondrial apoptosis-induced channel (MAC) in a voltage-dependent manner and is released into the cytosol during apoptosis (Guo *et al.* 2004). This initiates the apoptosome or caspase-activating complex to activate caspase 9, then caspases 7 and 3 (Green and Reed 1998). However, the exact role of this downregulated ubiquinol cytochrome c oxidoreductase subunit in the mitochondrial respiratory process of filarial nematode is not known. Uncharacterised bovine proteins belonging to the glutaminase and cytochrome P450 3A4 families, which were also upregulated in our findings, are proteins involved in metabolism. They are associated with respiration in xenobiotic

pathways (R-BTA-211981) and may signify a host response to the antibiotic treatment or to toxic products released by dying worms (Szklarczyk *et al.* 2015).

By association with NF (Armstrong *et al.* 2016), most of the upregulated worm proteins were excretory-secretory products with capabilities of regulating cell death. Cuticlin 1, an intra-uterine microfilaria (iuMf) protein of *O. ochengi* was first reported in *Caenorhabditis elegans* L1 to be essential for larval development (Sebastiano *et al.* 1991). Together with other forms (CUT3 and CUT5), they contain a zona pellucida protein domain of unknown exact molecular function (Sapio *et al.* 2005), but contribute to modelling worm shape at different stages of development. The upregulation of cuticulin and other collagen related proteins could be interpreted as an attempt by the worm to overcome external stress created by aggressive immune attack or internal stress resulting from the depletion of the endosymbiont *Wolbachia*. The upregulation of mitochondrial enzymes that regulate apoptosis included voltage-dependent-anion-channel-2 (nOo.2.0.1.t06017), which controls movement of metabolites in and out of the mitochondria (Rostovtseva and Bezrukov 2015), and thioredoxin peroxidases (Zhang *et al.* 1997). In addition, peroxiredoxin 6, a component of SHP (Armstrong and Benjamin unpublished), acts as an antioxidant (Fisher 2010), while disorganised muscle protein 1 (a vaccine candidate for *B. malayi*) stimultes a proinflammatory reaction (Kushwaha *et al.* 2014). There are several mechanisms that can lead to the activation of apoptosis, including extrinsic, intrinsic caspase-dependent (physiologic), or pathological caspase-independent mechanisms (Galluzzi *et al.* 2012).

Protein OVOC2486 is a novel uncharacterised protein containing six ShK-toxin domains (McNulty *et al.* 2015; Armstrong *et al.* 2016), and belongs to a unique group of filarial ShK-domain proteins which were first described from *L. sigmodontis*. In light of functional assays of nematode-derived ShK peptides, it probably exerts immunomodulatory activity by blocking Kv1.3 potassium channels in effector-memory T-cells (Chhabra *et al.* 2014). Most of the non-structural upregulated proteins were antioxidants, reflecting a worm response to reduce damage caused by ROS (Armstrong *et al.* 2016). However, these compensatory mechanisms did not prevent worm death after protracted chemotherapy.

Some organs of the filarial parasite may have been more affected than others by antibiotic therapy. Proteins of the cuticle and muscle tissues (cuticlin 1, gelsolin, laminin, cuticle collagen and muscle protein 1) were upregulated, while respiration-associated proteins were downregulated. All of the worm regulated proteins had previously been identified in intra-uterine microfilariae (Armstrong *et al.* 2016) and were shared proteins suggesting that post-ADT reduction in worm microfilarial load (Bah *et al.* 2015) contributed significantly to the worm regulated proteins observed. This also implies that the effect of adult worm degeneration was not detectable at T36. Antibiotic chemotherapy first affects the female reproductive organs causing sterility before worm mortality (Bandi *et al.* 1999; Gilbert *et al.* 2005; Hoerauf *et al.* 2008b; Supali *et al.* 2008; Landmann *et al.* 2011; Bah *et al.* 2014). The digestive system of *O ochengi,* which depends entirely on its bovine host for nutrition, is poorly developed (Buttner and Mac Donald 1985). There was no evidence of significant changes in digestive tract tissues. However, limited ingestion of fluid and blood does

occur during some stages of development of *Litomosoides sigmodontis* (Attout *et al.* 2005), while most nutrients may be absorbed directly via the endocytic compartment of the cuticle and by simple diffusion (Luck *et al.* 2016). Therefore, those changes in proteins of bovine origin observed which were not associated with immune processes around the worm, might be attributed to a decrease in feed intake. The reduction in blood component (Figure 4.7) observed was dominated by products of haemolysis of red blood cells and had very strong PPI with proteins involved in immune-related biological processes.

The main goal of searching for functional enrichments using only proteins that have significant FCs after antibiotic treatment was to highlight hidden biological processes that could facilitate the elucidation of mechanisms of worm death and thus potential new drug targets. We expected proteins from eosinophils to be upregulated and those from neutrophils to be downregulated (Hansen *et al.* 2011; Bah *et al.* 2015). The most upregulated protein was proteoglycan 3 (PRG3) precursor, an eosinophil major basic protein that regulates cytokine biosynthesis and activates neutrophil degranulation based on the Uniprot enzyme and pathway database (R-BTA-6798695). The top BLASTp hit to this protein in humans is to AAD24471.1 (prepro-major basic protein homolog) with 57% identity (Plager *et al.* 1999; Plager *et al.* 2001). The expression of this protein is highly restricted to eosinophils and basophils in humans (Plager *et al.* 1999), but in this study, we speculate it may have originated from eosinophils, which were significantly increased as observed from immunohistochemistry of extracted onchocercomata (Bah *et al.* 2015). A previous study reported insignificant changes in nodule basophils after antibiotic

chemotherapy (Nfon *et al.* 2006). STRING analysis, however, did not find significant biological processes or PPI associated with PRG3 precursor. This may be due to the limited conditions under which the protein is expressed (Plager *et al.* 1999). STRING is a bioinformatic tool that provides information on protein families, genes and biological processes in partnership with other databases that specialise in describing biological events based on curated or experimental evidences (Szklarczyk *et al.* 2015). It is however necessary that future studies should apply immunohistochemistry using anti-PRG3 (or antibodies against all the upregulated cattle proteins of uncertain origin) to locate the cells expressing PRG3 and other uncharacterised, regulated proteins within the onchocercomata. Such study should also ascertain whether the upregulated neutrophil proteins (BOLA-DRB3, Bota, MRC1, NCF4 and TAP1) involved in the phagosome processes correlated with PRG3. Nonetheless, KEGG pathway analysis suggests that the underlying mechanisms resembled those involved in herpes simplex infection, tuberculosis, allograft rejection, graft-versus-host, autoimmune thyroid disease, or type I diabetes mellitus pathways. These are chronic conditions causing increased apoptosis of neutrophils. The upregulated phagosome-related proteins (MRC1, TAP1, BOLA-DRB3, VIM and several other protein coded genes) regulate cell-mediated immunity. The common leucocytes involved in phagocyctosis are macrophages and neutrophils, and they are efficient in the control of bacterial and viral diseases, as well as in removing apoptotic cells or damaged tissue. Phagocytes can identify pathogens and then bind to engulf and inactivate them. Mannose receptor C type 1 protein (MRC1) recognises and binds to mannose, N-acetylglucosamine and fructose residues on the glycoproteins of pathogens. MRC1

also regulates the production of proteins that mediate endocytosis and neutralise engulfed pathogens within macrophages (Kim *et al.* 1992). The ABC transporting system (TAP1) found on the endoplasmic reticulum facilitates the transport of degraded cytosol-derived peptides generated by the proteasome. The TAP1 gene is co-expressed with Bota genes that encode the MHC class I receptors (Heise *et al.* 2016), while the BOLA-DB3 gene encodes the production of beta chain MHC class II proteins involved in antigen processing and presentation (Oprzadek *et al.* 2015). The MHC class II protein is a versatile protein with variants that affect susceptibility to various infections (Ford *et al.* 2009). Neutrophil cytosol factor 4 is a potent superoxidase (phagocyte NADPH oxidase) which is active during the respiratory burst (Matute *et al.* 2009).

Among the other upregulated bovine proteins, vimentin is an intermediate filament protein and component of the cytoskeleton that facilitates the maintenance of cell shape and cellular integrity (Yue *et al.* 2016). Both vimentin and S100 proteins are involved in the remodelling of neurofilaments and B-lymphocyte receptor signalling. Protein S100A4 is found as both a cytoplasmic and nuclear protein. It forms complexes with annexin A2 via double calcium binding to regulate several cellular processes during cell proliferation, migration and apoptosis (Mirsaeidi *et al.* 2016). One of the evidences of immune tolerance induced by filarial worm is the upregulation of tissue remodelling processes and associated proteins. Dihydropyrimidinase-related protein 3 (DPYSL3) is involved in cytoskeletal remodelling via interactions with class 3 semaphorins and plays a role in cell migration (Kanda *et al.* 2014). Its co-expression with fibrilin 1, an extracellular

glycoprotein that provides structural support within calcium-binding microfibrils in connective tissues is linked to a group of upregulated intranuclear and ribosomal proteins including lamin A/C; an inner nuclear envelope protein that mediates cell communication to promote cell growth by organisation of chromatins during cell division (Beck *et al.* 1990; Moriuchi *et al.* 2016). The co-expression with HNRNPM is justified by a weak experimental evidence (Lam *et al.* 2010). Cytoskeleton and intermediate filament proteins facilitate communication between the cytoplasm and outer membrane receptors via an uncharacterised ribosome-binding protein (LOC505941) situated on the outer membrane of the nucleus and endoplasmic reticulum. NHP2 like protein 1 is a component of the spliceosome intermediate C Complex (Morimoto and Boerkoel 2013) that contributes to the maturation of mRNA splicing initiated by HNRNPM..

Most regulated bovine proteins were observed to be downregulated in the current study. Cathepsin D (CTSD) is a protease that digests proteins within the highly-acidified lysosome. As a NF and SHP protein found outside of the lysosome, cathepsin D may influence cell signalling processes that prevent apoptosis (Deiss *et al.* 1996; Benes *et al.* 2008) The between-groups comparison of this study showed upregulation of CTSD precursor between SLT and CON, but time-course analysis showed significant downregulation between ADT at T0 and T36. CTSD precursor was co-expressed with upregulated vimentin and is an important constituent of SHP (Armstrong, Hetzel & Makepeace, unpublished). The depopulation of neutrophils and SHP (Gilbert *et al.* 2005; Nfon *et al.* 2006; Hansen *et al.* 2011) or NET (Tamarozzi *et al.* 2016) after antibiotic chemotherapy while eosinophils degranulate onto worm

cuticle are suggestive of a role in preventing the release of eosinophil chemoattractant essential for degranulation and worm killing.

4.5 Conclusion

The most depleted *Wolbachia* proteins were transmembrane proteins and PAL which have direct effect on neutrophil recruitment via TLRs. There were profound reductions in neutrophil-derived proteins such as cathelicidins, haptoglobin and defensin and proteins of bovine origin that depicted interruption of ingestion by worm. Downregulated worm proteins were excretory / secretory products, previously reported in NF and are known anti-inflammatory or immunoregulatory (galectin) and potential regulators of growth or apoptosis (amino dipeptidase). Most of the bovine proteins that adhered to worm surface were products of immune modulation associated with nodule fluid and SHP deposits with strong links to neutrophil degranulation. We predict that the ability of neutrophils to produce inflamatory cytokines suppressed in tolerance state was abrogated by upregulation of NGP after antibiotic therapy. An eosinophil major basic protein homologue was upregulated, thereby supporting the original hypothesis that worm death was due to depopulation of neutrophils while eosinophil degranulation increased. However, the complete depletion of both *Wolbachia* and neutrophils was not necessary to initiate the process of worm killing within the nodule.

Chapter 5 Transcriptomics of Oxytetracycline-treated *O. ochengi* within its Nodular Niche

5.1 Introduction

The transcriptomics of bovine onchocercomata (nodules) caused by *O. ochengi*, an intradermal parasite, was conducted for the first time. An onchocercoma is formed around an immobile, highly coiled and entangled female adult worm (Bwangamoi 1969) encapsulated within fibro-collagenous connective tissues richly supplied with blood capillaries (George *et al.* 1985). Male worms are smaller, motile and migrate from one nodule to another and may not be present in all nodules. Some mature females may contain spermatozoa from males that have migrated out (Hildebrandt *et al.* 2014). An onchocercoma can be easily isolated from the dermis and for this and other reasons, *O ochengi* is considered to be the best research model for human onchocerciasis (Trees 1992). However, extraction of intact female worms for studies is a challenge to overcome.

Although transcriptomics is perhaps best conducted on cells or tissue to facilitate interpretation of results, in this chapter, we processed entire nodules. The messenger RNA transcripts of any cell, tissue or an organism expressed at a given time is its transcriptome. The transcriptomics of onchocercomata will encompass transcriptomes of the worm, its endosymbiont (α-proteobacteria *Wolbachia*) and

bovine host tissues. WormBase ParaSite[1] is a freely accessible website of parasitic helminth genomes (Howe *et al.* 2016) that contains high-quality resources for *O. volvulus* using data generated by the Wellcome Trust Sanger Institute (WTSI) and New York Blood Center (Cotton *et al.* 2016), and on *O. ochengi* generated by the University of Edinburgh[2] and WTSI[3]. The *Onchocerca* parasites used for sequencing were obtained from Cameroon, where close to 9 million people are exposed to onchocerciasis (CAS 2012; Makepeace and Tanya 2016). The advent of post-genomic technology has rekindled the desire to identify biomarkers of filarial diseases and metabolic chokepoints that could enable drug repurposing and the monitoring of treatments (Luck *et al.* 2015; Armstrong *et al.* 2016; Bennuru *et al.* 2016). Transcriptomic studies generate huge quantities of data, but bioinformatic statistical tools are available to facilitate data processing and analyses to compare subjects under different treatments at the same time. Studies are ongoing to fine-tune the development of diagnostic tools that can differentiate infective larvae of *Onchocerca* spp. within the vector *Simulium* fly (Doyle *et al.* 2016) and identification of biomarkers for diagnosis of active disease or vaccine candidates with potential for disease control (Lustigman *et al.* 2017).

Since prolonged treatment with antibiotics of the tetracycline family was first shown to be macrofilaricidal by targeting intracellular *Wolbachia* in cattle and human *Onchocerca* spp. (Langworthy *et al.* 2000; Hoerauf *et al.* 2008b) and a gold-standard

[1] http://parasite.wormbase.org
[2] http://www.nematodes.org/genomes/onchocerca_ochengi/
[3] https://parasite.wormbase.org/Onchocerca_ochengi_prjeb1204/Info/Index

macrofilaricidal antibiotic protocol established using cattle *O. ochengi* in the early 2000s (Gilbert *et al.* 2005), the exact mechanism of worm killing is unknown. However, worm death is preceded by depletion of the endosymbiont *Wolbachia,* a reduction in the neutrophil population, and degranulation of eosinophils on the worm cuticle (Hansen *et al.* 2011). Hence, immunomodulation caused by the endobacteria has been proposed as the basis for symbiotic mutualism with the filarial parasite.

The endosymbiont of *Brugia malayi* (*w*Bm) belongs to *Wolbachia* supergroup D, while *w*Oo of *O. ochengi* belongs to supergroup C. Earlier genomic studies seeking the basis for *Wolbachia* mutualism with its filarial host using *B. malayi* proposed nutritional dependency, since the worm cannot synthesize haem and riboflavin (Elodie Ghedin *et al.* 2007) while the endosymbiont genome encodes the necessary pathways for these cofactors (Foster *et al.* 2005). However, other nematodes lacking symbionts, including the filarial nematode *Loa loa* (Desjardins *et al.* 2013), thrive despite a similar inability to synthesise haem and riboflavin (Rao *et al.* 2005). Strain *w*Oo cannot synthesise riboflavin as in *B. malayi*, but may generate energy in the form of ATP from purine biosynthesis to support the worm, particularly during embryogenesis (Darby *et al.* 2012). Recent transcriptome data from *w*Bm suggests that this may be in the case for *B. malayi* too (Grote *et al.* 2017). In *B. malayi*, immunolocalisation studies revealed *Wolbachia* surface protein *w*Bm00432 and wBm0152 in putative interactions with aldolase, enolase and other glycolytic processes to generate energy to the benefit of both the worm and bacteria (S.L. O'Neill *et al.* 2013; Voronin *et al.* 2016). Proteomic analysis of *w*Oo gene expression also confirmed the abundance of

Wolbachia immunogenic proteins that serve as ligands for mammalian TLRs (Darby *et al.* 2012) which is the basis for the immune mutualism, whereby the endosymbiont initiates pro-inflammation and attraction prolongation of neutrophils lifespan around worms, while *Wolbachia* continue to replicate within worms (Hansen *et al.* 2011).

The current study reports for the first time the *in vivo* effects of oxytetracycline treatment on the nodule transcriptome using the *O. ochengi* research model for human *O. volvulus*. Previously, gene analysis of oxytetracycline-treated female *B malayi* filarial parasites in the jird laboratory model for human lymphatic filaria (Ghedin *et al.* 2009) revealed downregulation of worm genes involved in energy metabolism and cuticle biosynthesis, and upregulation of those involved in protein synthesis following depletion of *Wolbachia* proteins. A follow up *in vivo* study on doxycycline-treated *B malayi* (Rao *et al.* 2012) confirmed efficacy of the drug against *Wolbachia* by interference with ribosome function, and highlighted trends in the worm response to treatment which were dominated by upregulation of pathways involved in survival mechanisms, suggesting why treatment needs to be protracted for macrofilaricidal efficacy. Analysis of gene expression in an *in-vitro* study of doxycycline-treated *Wolbachia* in an arthropod cell line using directional RNA-seq and label-free quantitative proteomics revealed upregulation of phosphate ABC transporter ATPase (Darby *et al.* 2014), well-known for increasing resistance to microbicides. This provides a further explanation for the necessity for protracted antibiotic therapy to effectively deplete *Wolbachia*.

The main objective of this chapter was to use transcriptomics to define the interplay between the worm, the bovine host and the *Wolbachia* symbiont over time and

during antibiotic chemotherapy to help determine the sequence of events that lead to worm death. The biological functions of the significantly expressed bovine genes may contribute to highlighting the environmental factors that precipitate worm death, as we expect bovine transcripts related to eosinophil activation to be upregulated and those associated with neutrophil proliferation to be downregulated. Changes within the worm will be analysed and linked with those observed in the endosymbiont *Wolbachia*. Meanwhile, the factors that enable the bacteria to tolerate treatment will be elucidated.

5.2 Materials and Methods

The experimental site, management of cattle and ethical considerations were described in Chapter 3 under methodology section (3.2.1). The samples processed and analysed in this chapter were a subset of those obtained from the main study experimental design described in Chapter 3, Section 3.2.3.1.

5.2.1 Experimental Animals and Design

Nine cows, three each from the adulticidal therapy (ADT), sublethal therapy (SLT) and untreated control (CON) groups were randomly selected from the seven available per group (Table 5.1). The cows were aged 6.26 ± 0.46 years by dentition and weighed 278.7 ± 6.9 kg on a weighing bridge (Marechalle™ PM 1000, France, ± 1 kg). Nodules collected at week 4 (T4), 8 (T8) and 52 (T52) post treatment were processed.

Table 5.1: Experimental block for transcriptomic study (total *N* = 27).

Treatment (number of cattle)	**Time T4**	**Time T8**	**Time T52**
ADT (n = 3)	ADT_T4_227	ADT_T8_227	ADT_T52_227
	ADT_T4_231	ADT_T8_231	ADT_T52_231
	ADT_T4_238	ADT_T8_238	ADT_T52_238
SLT (n = 3)	SLT_T4_222	SLT_T8_222	SLT_T52_222
	SLT_T4_226	SLT_T8_226	SLT_T5_226
	SLT_T4_240	SLT_T8_240	SLT_T52_240
CON (n = 3)	CON_T4_217	CON_T8_217	CON_T52_217
	CON_T4_236	CON_T8_236	CON_T52_236
	CON_T4_239	CON_T8_239	CON_T52_239
Sample size	9	9	9

5.2.2 Parasitology

5.2.2.1 *Nodulectomy, Handling and Evaluation of Worms in the Laboratory*

See Chapter 3, section 3.2.5.1 and 3.2.6

5.2.3 RNA Extraction and Sequencing

Onchocercomata were carefully dissected from the dermis to remove all contaminating tissues, weighed, preserved in five volumes of RNA*later* (Sigma), incubated at 4^{o}C overnight, and frozen at -20^{o}C for long term storage.

Before RNA extraction, each nodule was homogenized at the IRAD Wakwa Veterinary Laboratory facilities. All operations were done at room temperature in a laminar flow cabinet using QIAGEN® RNeasy MIDI or MAXI kits, following the manufacturer's procedures. Stored nodules in RNA*later* were removed, soaked in 0.5 mL RTL lysis-binding buffer solution in Petri dishes, and sliced into 1 - 2 mm^3 pieces. The contents were transferred into 7-mL sterile cryogenic tubes for homogenization. Nodules weighing less than (or more than) 75 mg were homogenized at maximum speed for 1 min in 2 (or 4) ml buffer RTL lysis-binding solution, respectively. Homogenization was performed to minimize foaming by reducing cycle time but increasing cycles of homogenization if required. To avoid cross-contamination of samples, the piston of the homogenizer was washed by running at maximum speed in 10% (v/v) Labtec detergent (Fisher Scientific), followed by rinsing in distilled water twice between each sample.

Debris and fat present in the homogenates were spun out (5,000 x g for 10 min) and the supernatant transferred into a 10-mL conical centrifuge tube. An equal volume

of 70% molecular graded ethanol was added, mixed gently to precipitate nucleic acid, and filtered through an RNeasy spin column (4 mL of the homogenate a time) at 5,000 x g for 5 min. Trapped nucleic acid on the silica-gel membrane was purified with wash buffer RW1, and DNA was digested with 160 µl DNase I incubation mixture (20 µl DNase I stock solution with 140 µl Buffer RDD), before washing again with buffer RW1 and twice with buffer RPE as described in the QIAGEN® RNeasy MIDI or MAXI protocol. The remaining purified RNA was eluted from the spin column with RNase-free water (100 µl for up to 75 mg and 150 µl for 76 - 150 mg tissues) twice into Qiagen-supplied RNase-free Eppendorf collection tubes. The RNase-free water was introduced directly on the membrane of the spin column and incubated for 1 min before centrifugation at 3,500 x g for 3 min. The eluted sample was split into two aliquots and immediately frozen at -80^{o}C. The samples were transported to Liverpool on dry ice where they were stored at -80^{o}C.

RNA quantification was done at room temperature using RiboGreen® fluorimetry (Invitrogen, Paisley, UK), following the manufacturers' protocol. Briefly, equal volumes of the standard rRNA or samples and RiboGreen® dye were mixed, incubated for 5 minutes in the dark, and ODs read at 520 nm on an Infinite™ 200 PRO multimode plate reader (Tecan, Männedorf, Switzerland). The integrity of the RNA to be submitted for sequencing was analysed using the Agilent RNA 6000 Nano reagent kit and the Agilent 2100 Bioanalyzer instrument (assay class Eukaryote Total RNA

Nano series II, version 2.5) as described by the manufacturer[4]. Only samples with at least 500 ng RNA in 26 µl with RIN scores >7.0 were sent to the Centre for Genomic Research (CGR) at the University of Liverpool for sequencing.

The concentration of RNA in each sample prepared for batch sequencing was standardised at 5 ng/µl. Only 24 of expected 27 samples (Table 5.1) were processed as a run due to poor RNA yield and quality for the three ADT_T52 samples. Prior to sequencing, prokaryotic and eukaryotic ribosomal RNA in the samples was depleted using Ribo-Zero™ RNA removal kits following the manufacturer's guidelines (Magnetic Gold Kit, Epidemiology; Illumina) and the RNA was purified by the ethanol precipitation method. RNASeq libraries were prepared using the strand-specific ScriptSeq™ v2 RNA-seq kit. The indexed libraries were paired-end sequenced by synthesis across seven lanes of Illumina HiSeq 2000 platform. Millions of mappable reads per lane were generated and the sequenced data subjected to QC, indexed, and saved as fastq files.

5.2.4 Sequenced Data Analysis and Bioinformatics

The sequence data were first directly mapped onto the Hereford (*Bos taurus*) reference cattle assembly[5] and GFF files[6]. Reads that did not match the cow reference were filtered out using a NGSUtils / bamutils library and mapped onto the *O. ochengi*

[4] http://rai.unam.mx/manuales/lbg_ARNGuideAgileny.pdf
[5] http://www.ncbi.nlm.nih.gov/assembly/GCF_000003055.6/FASTA
[6] ftp://ftp.ncbi.nih.gov/genomes/Bos_taurus/GFF/

genome[7], and finally, reads that mapped neither to cow nor *O. ochengi* were mapped to the *Wolbachia* symbiont of *O. ochengi* (*w*Oo) genome[8]. Mapping was done using Bowtie2 because the *Wolbachia* genome does not have introns and there was no need for splice-aware mappers like TopHat. The read outputs were recorded as BAM files and queried along with the cattle GFF[9], then the *O. ochengi* and the *Wolbachia* GFFs, treating all the reads as single-end. The number of reads falling within each *transcript* as annotated in the cattle, *O. ochengi* and *Wolbachia* GFFs (htseq-count parameter specification - --type transcript -i transcript_id -f bam) were counted using the htseq-count tool (Anders *et al.* 2015). The htseq-count produced a two-column text file for each dataset queried, the output files merged all the data files (104 in total), and the results were combined to produce a matrix-like representation in text form for each timepoint.

The sequenced datasets were named (10-227_T8_140313_L006_R1.fastq.gz) to incorporate cow ear tag number (in 200s) and timepoint tags (T4, T8 or T52) in weeks post-treatment (Table 5.1). Each dataset was processed individually. At the end, the results from individual datasets were combined to produce a matrix-like text format with transcripts and their observed expressions across various animals. Three such text files (T4.txt, T8.txt and T52.txt) were produced, each representing observations

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http://parasite.wormbase.org/Onchocerca_ochengi_prjeb1809/Info/Index, WBPS5

[8] ftp://ftp.ensemblgenomes.org/pub/bacteria/release-30/gff3/bacteria_19_collection/wolbachia_endosymbiont_of_onchocerca_ochengi/Wolbachia_endosymbiont_of_onchocerca_ochengi.GCA_000306885.1.30.gff3.gz

[9] ftp://ftp.ncbi.nih.gov/genomes/Bos_taurus/GFF/

for a time point (Table 5.1). All the codes used, data, and results files obtained were stored locally on the CGR cluster.

Differential expression (DE) analysis was carried out using the EdgeR package (Robinson *et al.* 2010) on bovine, *O. ochengi* and *Wolbachia* transcripts separately and pairwise comparisons between treatments and/or time points were conducted. Genes with low expression (cut-off value <5) were filtered out, the data normalized, and genes analysed for significant DE using the GLM approach with a fold change (FC) cut-off of ≥2 (equivalent to $\log_2$ FC ≥1) and a critical probability of $p < 0.01$. Transcripts of bovine origin with significant DE were group searched on String DB against the bovine genome (Szklarczyk *et al.* 2015) for protein interactions and identification of significant biological processes.

Genes with significant DE were subjected to Pfam enrichment (Pfam v. 30) analysis using the gathering threshold as a cut-off for functional enrichment as previously described (Armstrong *et al.* 2014). A hypergeometric test for enrichment of Pfam domains in the various sets of non-overlapping DE genes compared to the complete transcriptome was performed using R (phyper) (Falcon and Gentleman 2008), and corrected p values were obtained using the step-up false-discovery rate-controlling procedure (Benjamini and Hochberg 1995). Enrichment was considered statistically significant at $p < 0.01$.

5.3 Results

5.3.1 Onchocercomata Transcript

A total of 17,450 quantifiable gene transcripts were obtained from each of the 24 onchocercomata. Three rejected samples from the ADT group at T52 had low extracted RNA concentrations and consequently failed QC for sequencing. Two-thirds (68%) of the gene transcripts obtained were from the worm, *O. ochengi.* Twenty-eight percent were of bovine and 4% of *Wolbachia* origin. Table 5.2 summarizes the proportion of regulated (DE) gene transcripts counted with respect to source. After having removed duplicates from comparisons within fixed factors, between treatment groups, the number of transcripts expressed in both between treatment and time comparisons are reported. Note that substantial numbers of bovine (SLT) and worm (CON) transcripts were significantly regulated over time (Table 5.2). To ensure that only unique changes resulting from the antibiotic treatments were considered, a conservative approach was taken where overlaps in DE between longitudinal changes in the CON group and comparisons involving the SLT and ALT groups were identified and excluded from further analysis.

5.3.2 Bovine Transcripts from Onchocercomata

Bovine transcripts describe the worm's environment, particularly immune cells trapped between the vascularised nodule capsule formed principally of extracellular matrix and the interface with the worm(s). Eighteen percent (890) of 4,872 bovine transcripts quantified showed significant DE. One hundred and forty-six of (16.4%) the DE bovine transcripts were unannotated. The SLT treatment group accounted for most of the regulated transcripts both at T8 and T52 (Figure 5.1, A, C). However, the

highest number of upregulated genes in the SLT group was seen at T8 (Table 5.2). Fifty-two percent of the DE bovine genes were affected by both treatment and over time. These statistics of the bovine response to antibiotic treatment signify the important role of the host response to parasite establishment. To determine which treatment was responsible for the changes, the identity of the DE transcripts by treatment was considered.

Table 5.2: Summary statistics of quantifiable genes obtained from RNA Seq of bovine onchocercomata.

Statistical Test (ST)	Parameters		Transcribed genes count (GC) of bovine onchocercomata								
			Bovine origin			*O. ochengi*			*Wolbachia*		
	Fixed	Tested	Up↑	Down↓	Sample ^{N}	Up↑	Down↓	Sample ^{N}	Up↑	Down↓	Sample ^{N}
Between groups	T4	CON vs SLT	14	20	4750	130	32	11440	1	0	637
		CON vs ADT	0	8		19	23		3	0	
		SLT vs ADT	30	20		42	113		1	0	
	T8	CON vs SLT	56	17	4422	238	665	11595	11	9	638
		CON vs ADT	10	22		67	29		1	1	
		SLT vs ADT	3	19		548	60		2	4	
	T52	CON vs SLT	425	4	4386	24	86	11377	30	4	639
Between time	CON	T4 vs T8	21	45	4632	127	364	11386	2	2	639
		T8 vs T52	13	3		731	356		1	1	
		T4 vs T52	33	16		115	228		1	1	
	SLT	T4 vs T8	493	15	4618	34	72	11649	0	4	639
		T8 vs T52	15	9		56	34		39	12	
		T4 vs T52	434	7		42	18		22	6	
	ADT	T4 vs T8	6	11	4317	482	246	11356	6	3	637
DE GC significant in time and also groups ST			**454**			**1002**			**29**		
Grand Total			**870***		**4872**	**2357***		**11938**	**98***		**640**

*Total number of DE genes. Up↑ Upregulated GC; ↓ downregulated GC; ^{N} sample size. T; Time-points X weeks after start of treatments; CON, untreated control; SLT, sub-lethal oxytetracycline therapy; ADT, adulticidal oxytetracycline therapy.

5.3.2.1 *Effect of Ineffective Chemotherapy (SLT) on Bovine Transcripts*

Of the 870 bovine DE transcripts (Table 5.2), 666 (76.5%) of them were results from the SLT treatment. The most regulated SLT transcripts with respect to the ADT group are presented (Table 5.3). String analysis as described in (section 5.2.4) revealed significant enrichment in four predicted protein domains: intermediate filament proteins (IPR001664), keratin type II (IPR003054), gamma-aminobutyric acid A receptor/glycine receptor alpha (IPR006028), and SEA domain (IPR000082).

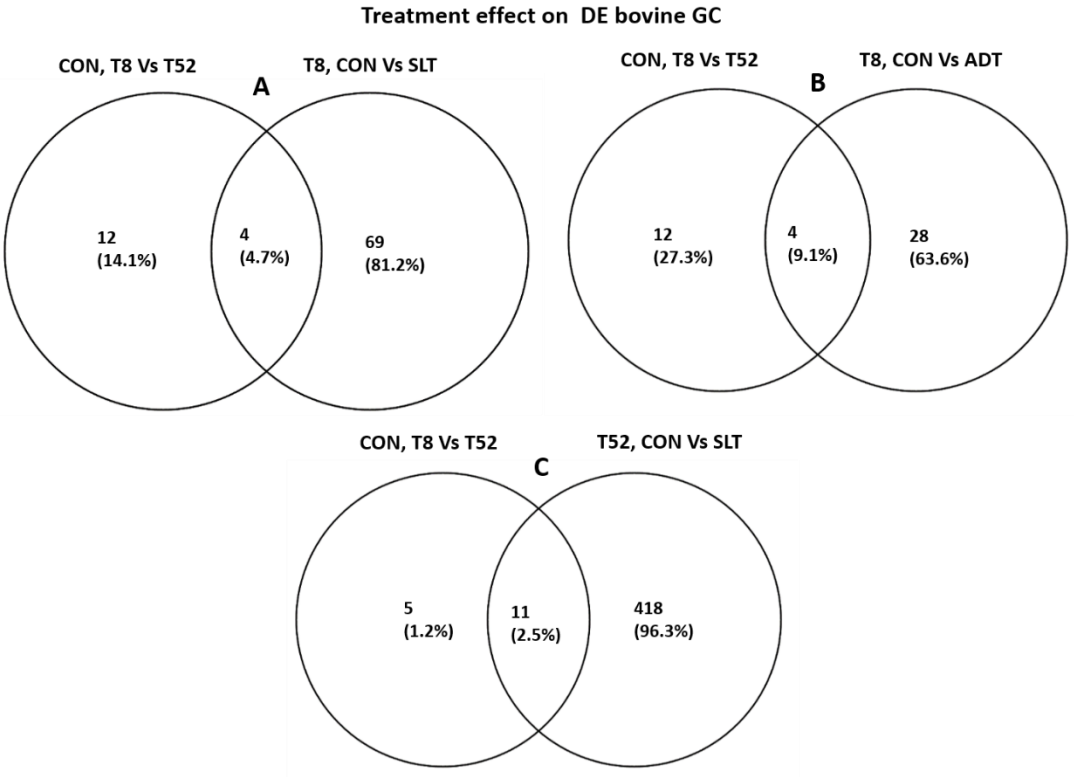

Figure 5.1: Overlap of DE bovine transcripts from treated onchocercomata with respect to T8 *vs*. T52 changes in the control group (P < 0.01, log2 FC ≥1). CON, untreated control; SLT, sublethal oxytetracycline therapy; ADT, adulticidal oxytetracycline therapy; at weeks T8 and T52 after first treatment. Venn sets represents GC overlaps for SLT at T8 (A) and at T52 (C), or ADT at T8 (B).

Most of the transcripts encoded cellular components of membrane transporter protein complexes (GO: 1902495) and are most likely represent neuroactive ligand-receptors (KEGG pathway: 04080) and extracellular ligand-gated ion channel activity (GO: 0005230) in immune cells. Enrichment in protein interactions were obtained only with the upregulated SLT bovine transcripts.

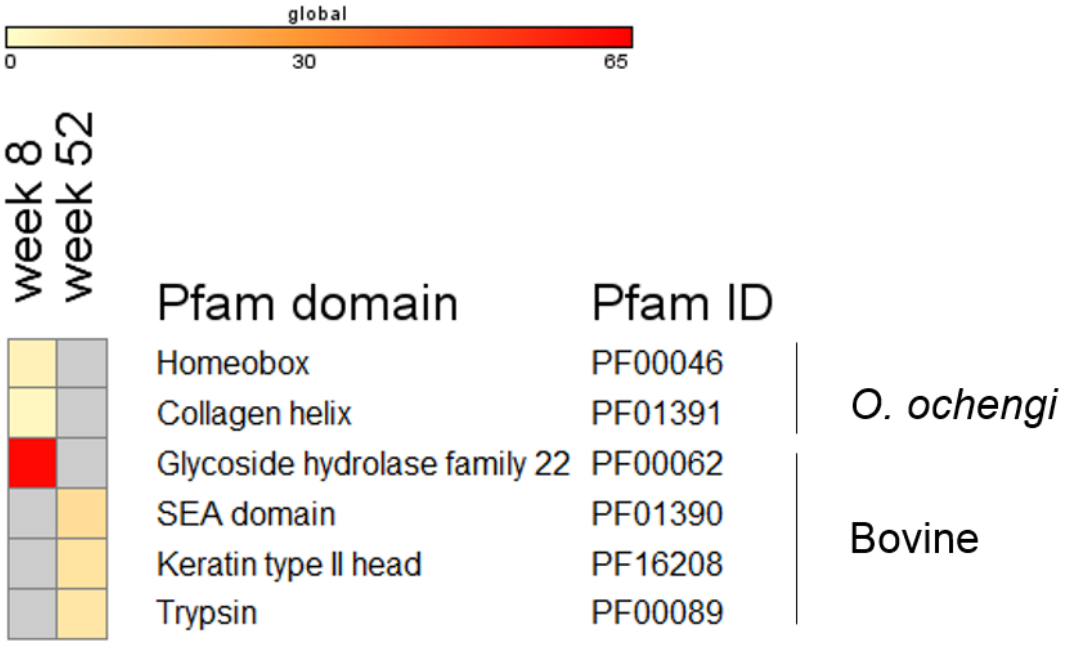

Figure 5.2: Functional enrichments associated with worm and cow DE genes at P <0.01. Legend scale 0 (grey) = unchanged and 65 (red) = highest significant enrichment

At T52, the vast majority of DE transcripts in the SLT group were upregulated (Table 5.2), with significant enrichment in those coding for extracellular protein domains (SEA domain), intermediate filament proteins (keratin type II head) involved in post translational modification (Stewart 1990), and trypsin. Transcripts with the greatest FC were predicted multidrug resistance-associated protein 4 (LOC100294973) and protein 4-like (LOC100847574) (Table 5.3). They were downregulated at T8 from T4

and upregulated at T52 from T8. However, apolipoprotein F (APOF) and partial mRNA component of multidrug resistance-associated protein 4-like (LOC101902112) were initially upregulated at T8 before downregulation at T52. APOF is an inhibitor in the metabolism of plasma high density cholesterol of molecular weight 29 kDa (Day *et al.* 1994; Lagor *et al.* 2012). Meanwhile, predicted multidrug resistance-associated proteins (PMRP) are members of ABC cassette transporter proteins associated with xenobiotic resistance (Keppler 2011).

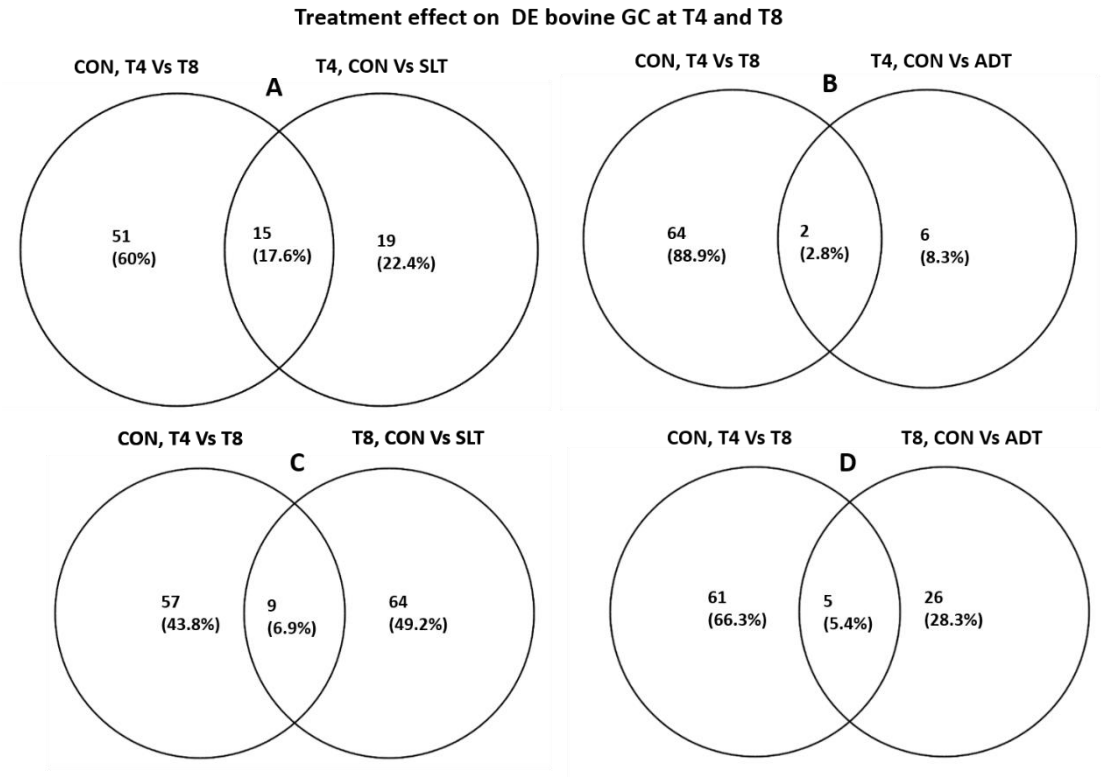

Figure 5.3: Overlap of DE bovine transcripts from treated onchocercomata with respect to T4 *vs* T8 changes in the control group (P <0.01, log2 FC ≥2). CON, untreated control; SLT, sublethal oxytetracycline therapy; ADT, adulticidal oxytetracycline therapy; at weeks T8 and T52 after first treatment. Venn sets represents GC overlaps for SLT at T4 (A) and at T8 (C), or ADT at T4 (B) and T8 (D).

Table 5.3: Most highly regulated bovine transcripts by SLT at T8 and T52.

UNIPROT ID	Gene Annotation	Log_2 FC			
		P-value	T4→T8	T8→T52	T4→T52
NM_001128502.2	Apolipoprotein F (APOF)	0.000	8	-5	-5
XM_010800734.2	Predicted: Multidrug resistance-associated protein 4-like (LOC101902112), partial mRNA	0.000	8	-5	-5
XM_010800691.2	Predicted: Multidrug resistance-associated protein 4 (LOC100294973)	0.000	-8	10	10
XM_010800692.1	Predicted: Multidrug resistance-associated protein 4-like (LOC100847574)	0.000	-11	9	9
NM_001038682.1	Activation-induced cytidine deaminase (AICDA)	0.000	7	NS	6
XM_015473328.1	Predicted: Uncharacterized LOC107132214	0.001	5	NS	7
NM_001098379.1	Interleukin 22 (IL22)	0.000	7	NS	NS
NM_001099703.2	Ureidoimidazoline (2-oxo-4-hydroxy-4-carboxy-5-) decarboxylase (URAD)	0.000	7	NS	NS
NM_001080339.1	Lysozyme (renal amyloidosis) (LYZ1)	0.001	7	NS	4
XM_002689805.3	Predicted: Family with sequence similarity 205, member A (FAM205A), transcript variant X1	0.001	NS	NS	7
NM_001025348.1	Serine peptidase inhibitor, Kazal type 1 (SPINK1)	0.001	7	NS	NS
XM_010809892.2	Predicted: fasciculation and elongation protein zeta 2 (FEZ2), transcript variant X1	0.000	7	NS	7
NM_001098385.1	Keratin 4 (KRT4)	0.001	7	NS	NS
XM_015472396.1	Predicted: Serine protease 55 (LOC783279)	0.001	7	NS	NS
NM_001114522.1	Serine peptidase inhibitor, Kazal type 4 (SPINK4)	0.002	7	NS	NS
XM_002690003.4	Predicted: Histone H2B type 1-L (LOC523702)	0.001	7	NS	NS
XM_010807764.2	Predicted: Interferon omega-1 (LOC618859)	0.000	7	NS	NS
NM_001046104.2	L-threonine dehydrogenase (TDH)	0.000	7	NS	NS
XM_002689672.4	Predicted: Protein FAM205A (LOC785500)	0.000	7	NS	NS
XM_010806616.2	Predicted: Transmembrane protein 221 (TMEM221), transcript variant X2	0.001	7	NS	NS
XM_010805276.2	Predicted: Olfactory receptor 6C68-like (LOC100140428)	0.002	NS	NS	6
XM_015471660.1	Follicular dendritic cell secreted protein (FDCSP)	0.001	6	NS	NS
NM_001192528.1	Cartilage intermediate layer protein 2 (CILP2)	0.001	NS	5	6
XM_010801560.1	Retinol binding protein 2 (RBP2), transcript variant X1	0.003	6	NS	NS
XM_002689303.4	Predicted: Double-headed protease inhibitor, submandibular gland (LOC100296105)	0.001	NS	NS	6
XM_005200930.3	Putative ubiquitin carboxyl-terminal hydrolase 17-like protein 23 , partial mRNA	0.003	6	NS	NS
XM_002688425.4	Predicted: Cytokine like 1 (CYTL1)	0.003	6	NS	NS
XM_010807205.1	Uncharacterized LOC101906185 (LOC101906185)	0.003	6	NS	6
XM_015471955.1	Uncharacterized LOC101906155 (LOC101906155)	0.005	6	-5	-5
NM_001193068.1	Keratin 86 (KRT86)	0.001	6	NS	NS
NM_001113727.1	Cationic trypsin (LOC780933)	0.002	6	NS	NS
XM_005202243.3	Predicted: Histone H2A type 1 (LOC524236)	0.001	6	-6	-6
NM_175773.3	Joining chain of multimeric IgA and IgM (JCHAIN)	0.004	NS	NS	-2
NM_001076259.2	Pentraxin 3 (PTX3)	0.006	-3	NS	NS
XM_005212042.3	Predicted: Iron-sulfur cluster assembly 2 (ISCA2), transcript variant X1	0.004	-3	NS	NS
XM_015474149.1	Predicted: Collagen type VI alpha 5 (COL6A5), transcript variant X1	0.007	NS	NS	-3
XM_015472370.1	Predicted: Family with sequence similarity 174 member A (FAM174A), transcript variant X1	0.006	-4	NS	NS
XM_015472829.1	Predicted: Single-minded family bHLH transcription factor 1 (SIM1), transcript variant X1	0.009	NS	NS	-5

Value of Log_2 fold change (FC) of DE transcripts at week four to (→) week eight (T8) or 52 (T52) and from T8 to T52. NS not significant.

Removing DE transcripts found in the untreated control over time from changes observed in the treatment groups (Figure 5.1), significant enrichment in glycoside

hydrolase family 22 (PF00062.18) at T8; and in trypsin (PF00089.24), keratin type II head (PF16208.3) and SEA (PF01390.18) protein domains at T52 (Figure 5.2) was apparent. The upregulation at T8 (an important turning point in worm recovery (Gilbert *et al.* 2005) of the glycoside hydrolases proteins may be an important indicator of the determinant role it plays in enhancing protective mechanisms for worm recovery and survival. While the trypsin, keratin and SEA proteins are evidence of establishment of normalcy in the intranodular milieu.

Homeobox domain genes were amongst the upregulated transcripts in between-group comparisons. They are known regulators of growth associated with repair of injuries, particularly angiogenesis in adults (Kachgal *et al.* 2012) and embryonic cell differentiation during early developmental processes (Sharma *et al.* 2013). Homeobox NKX2-6 (XM_003586411.1), fibroblast growth factor (FGF) 5 and NK2 homeobox 6, were among the upregulated transcripts at T8, though not amongst the top most regulated, suggesting a probable reconstruction of damaged vessels from differentiating stem cells (Kachgal *et al.* 2012; Wang *et al.* 2015). Interleukin 1 family member 10 (theta), protein HP-25 homologs 1 and 2, serine peptidase inhibitor, Kazal types 1 and 4, and interferon omega-1 are probably neutrophil transcripts that were upregulated at T8.

Early effects of antibiotics on bovine component of the nodule were discernible at T4 by contrasting treatment against untreated control in pairwise comparison of SLT, ADT and CON (Figure 5.3). Although both antibiotic-treated groups had received the same dose of oxytetracycline at T4, the response to SLT and ADT was quite different

(Table 5.4). SLT upregulated immunoregulatory transcripts (chemokines C-X-C, leukotrienne) and tissue remodelling transcripts such as ASTL transcript variant 1, known to contribute in embryonic development (Acloque *et al.* 2012) as early as T4 (Table 5.4).

Proteins that regulate cell-mediated immunity such as leukotriene B4 receptor 2 (LTB4R2) transcript variant X1 (Crooks and Stockley 1998), solute carrier family 5 member 7 (SLC5A7), and multidrug resistance-associated protein 4 (LOC100294973) were amongst the most downregulated at T4 but upregulated at T8 or T52 (Table 5.3).

5.3.2.2 *Effect of Macrofilaricidal Chemotherapy (ADT) on Bovine Transcripts*

The effects reported here depend on observations recorded at T4 and T8, because T52 was excluded for sequencing. Results of the most regulated transcripts from longitudinal comparisons and between treatment and control are presented (Table 5.5). At T8, only six transcripts were significantly upregulated and 11 downregulated in the longitudinal comparison (Table 5.2). However, only two transcripts were DE relative to the CON group at T8 amongst the 32 between-group significant changes (Table 5.5). The expression of voltage-dependent anion-selective channel protein 1 (VDAC1) at T8, though the highest compared to T4 (Table 5.5), was not significantly different from that of the CON. VDAC1 is a mitochondrial outer membrane channel protein through which ATP is exported out of mitochondria (Choudhary *et al.* 2014). It regulates cell volume and apoptosis and is a biomarker for mitochondrial dysfunction (Palmeira *et al.* 2014).

Table 5.4: Treatment effects differences between the ADT and CON on bovine transcripts at week four (T4)

UNIPROT ID	Gene Annotation	Log_2 FC at T4		
		P-Value	SLT→ADT	CON→SLT
XM_010800692.1	Predicted: Multidrug resistance-associated protein 4-like (LOC100847574)	0.000	10	-10
XM_010800691.2	Predicted: Multidrug resistance-associated protein 4 (LOC100294973)	0.001	7	-9
NM_001098469.2	ATP-binding cassette transporter C4-like (LOC617079)	0.003	6	NS
XM_015473029.1	Predicted: Leukotriene B4 receptor 2 (LTB4R2), transcript variant X1	0.000	5	-5
NM_001101924.1	TRPM8 channel associated factor 2 (TCAF2)	0.000	3	-4
NM_001034447.2	Fructose-bisphosphatase 1 (FBP1)	0.001	3	-3
NM_001192167.1	Solute carrier family 28 (concentrative nucleoside transporter), member 3 (SLC28A3)	0.000	3	-3
XM_015472616.1	Predicted: SLC28A3, transcript variant X2	0.000	3	-3
XM_010810101.2	Predicted: Antimicrobial peptide NK-lysin-like (LOC104968634)	0.005	3	NS
NM_001025325.2	Placenta-specific 8 (PLAC8)	0.006	3	NS
NM_001034738.1	Leukotriene B4 receptor (LTB4R)	0.003	3	NS
NM_180999.1	Lysozyme C-2 (LYZ2)	0.002	2	-3
NM_001078098.1	RELT like 1 (RELL1)	0.001	2	-2
NM_001101062.1	Platelet factor 4 (PF4)	0.003	2	-2
NM_001206785.1	Immunoglobulin superfamily, member 10 (IGSF10)	0.001	2	-2
XM_003585693.4	Predicted: Succinate receptor 1 (SUCNR1)	0.000	2	-3
NM_001113172.1	C-X-C motif chemokine ligand 9 (CXCL9)	.0.001	2	NS
NM_173968.3	Thioredoxin (TXN)	0.002	1	-1
XM_002691130.5	Predicted: Astacin-like metallo-endopeptidase (M12 family) (ASTL), transcript variant X1	0.007	NS	5
XM_010805731.2	Predicted: Solute carrier family 16 (monocarboxylate transporter), member 8 (SLC16A8)	0.009	NS	5
XM_002689143.2	Predicted: Proteinase 3 (PRTN3)	0.008	NS	5
NM_174299.3	Chemokine (C-X-C motif) ligand 2 (CXCL2)	0.004	NS	3
XM_010810565.2	Predicted: Laminin subunit gamma 3 (LAMC3), transcript variant X1	0.004	-2	2
XM_003586355.4	Predicted: Uncharacterized LOC100848912 (LOC100848912)	0.009	-3	NS
NM_001105006.1	Lipase I (LIPI)	0.004	-3	NS
XM_010807215.2	Predicted: Olfactory receptor 2Z1 (LOC100299275), transcript variant X1	0.010	-4	NS
XM_015471483.1	Transmembrane protein 155 (TMEM155), transcript variant X1	0.008	-4	NS
XM_015472034.1	Predicted: Chromosome 7 open reading frame, human C19orf45 (C7H19orf45)	0.003	-5	NS
XM_005202243.3	Predicted: Histone H2A type 1 (LOC524236)	0.005	-5	NS
XM_002687050.4	Predicted: Crystallin gamma N (CRYGN)	0.006	-6	NS
XM_002688707.2	Predicted: Olfactory receptor, family 7, subfamily A, member 10-like (LOC789504)	0.008	-6	NS
XM_002683645.3	Predicted: Olfactory receptor 2AJ1 (LOC614592)	0.006	-6	NS
NM_001192528.1	Cartilage intermediate layer protein 2 (CILP2)	0.002	-6	NS
XM_015471660.1	Predicted: Follicular dendritic cell secreted protein (FDCSP)	0.004	-6	NS
XM_005211689.3	Predicted: C2 calcium-dependent domain containing 4B (C2CD4B), transcript variant X1	0.004	-6	NS
NM_175817.3	Fatty acid binding protein 1 (FABP1)	0.008	-6	5
XM_010801760.2	Predicted: Methyltransferase like 6 (METTL6), transcript variant X3	0.003	-6	5
NM_001001148.1	Gastrokine 1 (GKN1)	0.006	-7	5

NS, not significant difference; log_2 FC, Log transformed fold change; → direction of FC

Comparing transcript expression at T8 to those of CON, predicted multidrug resistance-associated proteins 4 (LOC100294973 and LOC100847574) expression was

significantly elevated in the ADT-treated nodules (Table 5.5) similar to that at T4 (Table 5.4), implying that the response was already elevated at T4 and remained high through T8. Interestingly, in the SLT group it was downregulated. Multidrug resistance-associated protein 4 gene is either a protein coding gene or an intron (Zimin *et al.* 2009) belonging to sub-family C of ATP-binding cassette transporters. It is associated with resistance to antimicrobials (Keppler 2011; Dai *et al.* 2017). While histone H2B type 1-L (LOC523702) was upregulated at T8 by SLT (Table 5.3), histone H2A type 1 (LOC524236) was downregulated at T4 by both ADT and SLT (Table 5.4). These changes might reflect an increase in mitochondrial activity, energy metabolism, and oxytetracycline efflux from bovine cells, and mitochondrial stress caused by long-term tetracycline exposure.

The most downregulated bovine transcripts by ADT treatment (Table 5.5) were membrane transporter proteins, signifying a reduction in the regulation of multiple cellular processes associated with drug metabolism (Fernández-Calotti *et al.* 2016), angiogenesis or tissue repair (FGF23), and the ability to regulate phosphate and vitamin D metabolism (Lanske and Razzaque 2014). Immune effectors such as T-cell receptor alpha chain V region HPB-MLT, ACTG2 and NKG2D ligand 4 or lymphocyte effector toxicity activation ligands were downregulated. NKG2D is one of the natural cytotoxicity receptors which allow natural killer cells to participate in the primary immune reaction to pathogens (Haik et al. 2016). Homeodomain transcripts were less expressed in the ADT than in the CON group.

Table 5.5: ADT regulated transcripts of bovine origin at 8 weeks after start of treatment

UNIPROT ID	Annotation	Log_2 FC		
		P-Value	ADT-T8/T4	At T8-ADT/CON
XM_005209337.1	Voltage dependent anion channel 1 (VDAC1), transcript variant X1	0.008	3	NS
NM_001193108.1_2	#N/A	0.003	1	2
XM_002688466.4	Bone marrow stromal cell antigen 1 (BST1)	0.008	1	NS
XM_015471786.1	Sortilin-related VPS10 domain containing receptor 2 (SORCS2)	0.009	1	NS
NM_001015669.1	BMP/retinoic acid inducible neural specific 1 (BRINP1)	0.009	1	NS
XM_010809545.1	Synaptotagmin 16 (SYT16)	0.009	1	NS
NM_177506.2	Glutaminyl-peptide cyclotransferase (QPCT)	0.006	-1	NS
NM_001034419.2	Hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD)	0.006	-1	NS
NM_001045901.1	Growth arrest and DNA damage inducible gamma (GADD45G)	0.006	-1	NS
XM_010805460.2	Single pass membrane protein with coiled-coil domains 3 (SMCO3), transcript variant X1	0.001	-2	NS
NM_174603.3	Solute carrier family 2 member 3 (SLC2A3)	0.004	-2	NS
XM_015473641.1	MX dynamin-like GTPase 2 (MX2), transcript variant X1	0.007	-2	NS
XM_002687880.3	Fibroblast growth factor 23 (FGF23)	0.003	-2	NS
NM_001075961.1	Receptor transporter protein 4 (RTP4)	0.000	-2	NS
NM_001083519.1	Inositol-trisphosphate 3-kinase A (ITPKA)	0.004	-2	NS
NM_001192167.1	Solute carrier family 28 (concentrative nucleoside transporter), member 3 (SLC28A3)	0.005	-2	-2
NM_001192578.2	TOP1 binding arginine/serine rich protein (TOPORS)	0.008	-3	NS
XM_010800691.2	Multidrug resistance-associated protein 4 (LOC100294973)	0.000	NA	9
XM_010800692.1	Multidrug resistance-associated protein 4-like (LOC100847574)	0.000	NA	9
XM_005202243.3	Histone H2A type 1 (LOC524236)	0.007	NA	4
XM_010801870.2	#N/A	0.010	NA	2
NM_001193108.1_2	#N/A	0.000	NA	2
XM_002690745.5	Purine nucleoside phosphorylase (LOC790312)	0.005	NA	2
NM_001035040.2	RAB19, member RAS oncogene family (RAB19)	0.002	NA	-2
NM_001034302.2	SPARC like 1 (SPARCL1)	0.001	NA	-2
NM_001206810.1	Thrombospondin-type laminin G domain and EAR repeats (TSPEAR)	0.004	NA	-2
XM_015473259.1	Tetratricopeptide repeat domain 9 (TTC9), transcript variant X1	0.009	NA	-2
NM_183362.1	Resistin (RETN)	0.003	NA	-2
NM_001102084.1	Neural EGFL like 2 (NELL2)	0.005	NA	-2
NM_001013592.1	Actin, gamma 2, smooth muscle, enteric (ACTG2)	0.000	NA	-2
XM_003586424.4	BARX homeobox 1 (BARX1)	0.010	NA	-2
XM_015472906.1	NKG2D ligand 4 (LOC100848282)	0.007	NA	-3
XM_010809066.2	T-cell receptor alpha chain V region HPB-MLT (LOC786808)	0.000	NA	-6

NS = not significant; NA = not applicable; #N/A = Annotation note found; ADT T8/T4 FC at eight weeks from T4 within adulticidal therapy group. At T8 ADT/CON difference in transcript expression between ADT from the untreated control.

The differences between the effects of SLT to those of ADT will enable a better identification of transcripts that depict resistance or recovery from treatment. Histone H2A type 1, gastrokine 1 and METTL6 were amongst the 14 most downregulated by ADT when compared to SLT (Table 5.4). Meanwhile, the level of

expression of multidrug resistance-associated protein 4-like, ATP-binding cassette transporter C4-like (LOC617079), leukotriene B4 receptor antimicrobial peptide NK-lysin-like (LOC104968634), lysozyme C-2 (LYZ2) and C-X-C motif chemokine ligand 9 (CXCL9) were higher than in the SLT group. CXCL9 is a ligand to CXCR3 of macrophages which together with IL-2 stimulates the activation of cytotoxic lymphocytes (Pan *et al.* 2006) and eosinophilic degranulation on worms (Turner *et al.* 2018; Zhang *et al.* 2018). This could signify that there was activation of the monocyte-macrophage system to enhance the activity of cell-mediated immunity of the ADT, while regulators of tissue homeostasis and repair were upregulated by SLT.

5.3.3 *Onchocerca ochengi* Transcripts

The worm contributed the highest number of transcripts (11,938) from the onchocercoma (Table 5.2). Twenty percent (2,357) of the worm transcripts were significantly regulated and about 80% (1,721) were from the untreated control group (Table 5.2). Figure 5.4 illustrates that the relationship between longitudinally-regulated GC of the untreated control to those of the different treatments. At T8, the SLT had the highest number of regulated transcripts in between-group comparisons (Figure 5.4), most of which were significantly regulated in CON at T52. At T8, the ADT worms had more than 500 upregulated genes in a pair-wise comparison with SLT, and this accounted for more than within-ADT group upregulated transcripts (Appendix 0; Table 5.2).

Pfam analysis of the non-redundant *O. ochengi* transcripts revealed enrichments in homeobox domain and collagen helix gene families only at T8 (Table 5.2). Half of the

DE worm transcripts were uncharacterised, hypothetical, or novel proteins with no description.

5.3.3.1 *Ineffective Chemotherapy (SLT): Effects on Worm Transcripts*

Cuticle collagen 6 or ROL-6 was upregulated at T8 (log FC = 6, p <0.01), while cullin-1-like isoform 1 (log FC = 8, p <0.01) and collagen alpha-2 chain precursor (log FC = 8, p <0.01) were downregulated at T52. Others amongst the most downregulated genes at T52 (FC >8) were macrophage receptor MARCO (nOo.2.0.1.t11349-RA), antigen maltose binding protein (nOo.2.0.1.t13053-RA) and prolyl 4-hydroxylase (nOo.2.0.1.t12403-RA).

Among twenty most-upregulated (FC > 6), worm transcripts both at T8 and T52, only four have a description: presenilin spe-4 (nOo.2.0.1.t13339-RA), zinc knuckle family protein (nOo.2.0.1.t13328-RA), glutamate dehydrogenase (nOo.2.0.1.t11374-RA) and receptor type guanylyl cyclase (nOo.2.0.1.t04747-RA). Heat shock protein 60 (nOo.2.0.1.t09027-RA), DNA mismatch protein (nOo.2.0.1.t13793-RA), deoxyribonuclease TatD (nOo.2.0.1.t07516-RA) and a novel protein (nOo.2.0.1.t12987-RA) were upregulated at T52 but downregulated at T8. It is most likely that these genes make an important contribution to worm survival.

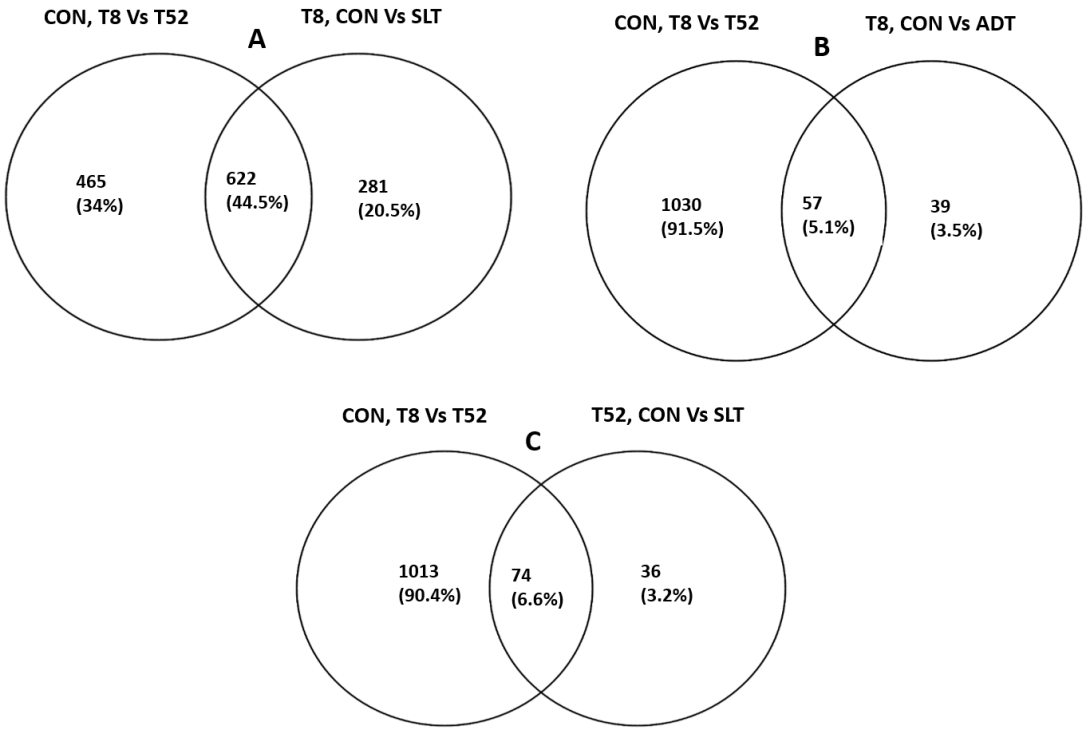

Figure 5.4: Overlap of DE *O. ochengi* transcripts from treated onchocercomata with respect to T8 *Vs* T52 changes in the control group (P < 0.01, irrespective of log2 FC≥2). CON, untreated control; SLT, sublethal oxytetracycline therapy; ADT, adulticidal oxytetracycline therapy; at weeks T8 and T52 after first treatment. Venn sets represent GC overlaps for SLT at T8 (A) and at T52 (C), or ADT at T8 (B).

At T4, glutamate dehydrogenase, nematode cuticle collagen n-terminal domain containing protein (nOo.2.0.1.t04025-RA), three cuticlin 1 transcripts, homeobox-domain containing protein (nOo.2.0.1.t02253-RA) and homeotic protein spalt-major (nOo.2.0.1.t10063-RA) were among the transcripts upregulated by SLT against CON. The most downregulated (FC>5) were uncharacterised genes alongside polyubiquitin-a-like isoform 1 (nOo.2.0.1.t12148-RA), a hypothetical protein similar to LOAG_09784 [*Loa loa*], major intrinsic protein (nOo.2.0.1.t08639-RA), circadian locomoter output cycles protein kaput-like (nOo.2.0.1.t05447-RA) and phosphatidate cytidylyltransferase (nOo.2.0.1.t07510-RA).

5.3.3.2 *Macrofilaricidal Effects (ADT) on Worm Transcripts*

Macrofilaricidal therapy upregulated 482 and downregulated 246 worm genes between T4 and T8, while 548 transcripts were upregulated when compared with SLT at T8 (Table 5.2). Amongst the 35 most upregulated genes at T8 in ADT (FC >6) were antigen maltose-binding protein (nOo.2.0.1.t12597-RA), collagen alpha-1 chain-like (nOo.2.0.1.t12681-RA), cre-tag-297 protein (nOo.2.0.1.t11709-RA), amidophosphoribosyl transferase (nOo.2.0.1.t13865-RA), nematode cuticle collagen n-terminal domain containing protein (nOo.2.0.1.t10208-RA), HSP 60, cuticlin 1, cytochrome c oxidase subunit I, and cell death specification proteins 1 (nOo.2.0.1.t01803-RA) and 2 (nOo.2.0.1.t06269-RA). Cell death specification protein, (CES)-1 and CES-2 are regulators of programmed cell death in neurons (Wang *et al.* 2006). Instead, cuticle collagen 6 (nOo.2.0.1.t12333-RA) and homeobox protein dlx-1 (nOo.2.0.1.t04757-RA) were downregulated. Although antigen maltose-binding protein (nOo.2.0.1.t12597-RA) had the most significant within-group time effect at T8 and between-group comparison with CON at T4, it was not significantly different to the control at T8. This implies that there was a similar increase of the gene expression within the untreated control group over time.

Between-group comparisons of ADT to SLT revealed tenfold upregulation of macrophage receptor MARCO (nOo.2.0.1.t11349-RA) and six other novel transcripts (nOo.2.0.1.t08607-RA, nOo.2.0.1.t10492-RA, nOo.2.0.1.t08121-RA, nOo.2.0.1.t13462-RA, nOo.2.0.1.t10494-RA and nOo.2.0.1.t11104-RA). Other highly upregulated transcripts at T8 were antigen maltose-binding protein, prolyl 4-hydroxylase, cre-tag-297 protein, CES 2, nematode cuticle collagen n-terminal

domain containing protein and many other uncharacterised transcripts. These genes, except HSP 60, were downregulated by the SLT.

5.3.4 *Wolbachia* Endosymbiont Transcripts from Bovine Onchocercomata

Wolbachia had the least number of transcribed genes (640) quantified from bovine onchocercomata (Table 5.2), although this represents expression from 99% of the intact genes in the tiny *w*Oo genome (Darby *et al.* 2012). Ninety-seven (15%) genes had significant DE, of which 29 were significant outcomes from both the between-treatments and time comparative statistics (Table 5.2). The greatest number of regulated *Wolbachia* transcripts (Table 5.2), accounting for about 59 % of its regulated genes, were from the SLT group. List of upregulated *Wolbachia* transcripts is attached (Appendix 0).

5.3.4.1 *Effects of Ineffective Antibiotic Chemotherapy (SLT) on Wolbachia*

The highest numbers of upregulated genes from SLT *Wolbachia* transcripts were between T4 and T52 (51), and the most regulated are presented (Table 5.6). The first significant process resulting from SLT was characterised by downregulation of transcripts associated with aerobic respiration at T8 (Figure 5.5B, Table 5.6), such as cytochrome c oxidase (cox11) and ferredoxin. Cox11 is a mitochondrial respiratory chain complex component (KEGG ko00190) required for copper binding. Downregulated pre-protein translocase, an internal membrane protein regulator and zinc-binding DnaK suppressor genes (Figure 5.5) are key evidence of the early response to antibiotic-induced stress geared at conservation of energy. At T4, only one hypothetical protein's transcript (wOo_08690) was upregulated (Figure 5.5).

Table 5.6: Transcripts most regulated by SLT at T8 and T52

GENE ID	log_2 FC	P-value	Gene Annotation	Differential Expression		
				T4→T8	T4→T52	T8→T52
wOo_05670	7	0.000	Phosphatase	NS	**UP**	NS
wOo_08750	7	0.000	ABC type Mn2+ Zn2+ transport system permease component	NS	NS	**UP**
wOo_10500	7	0.000	30S ribosomal protein S10	NS	NS	**UP**
wOo_03860	6	0.000	Putative translation factor SUA5	NS	NS	**UP**
wOo_00220	6	0.000	tRNA Ser GCT	NS	**UP**	NS
wOo_02750	6	0.001	Cytochrome B561	NS	NS	**UP**
wOo_07890	6	0.003	Hypothetical protein	NS	**UP**	NS
wOo_09200	6	0.002	NADH ubiquinone oxidoreductase 18 kDa subunit	NS	NS	**UP**
wOo_01370	6	0.003	N6 adenine specific methylase	NS	NS	**UP**
wOo_05560	6	0.003	RimM protein required for 16S rRNA processing	NS	NS	**UP**
wOo_00840	6	0.006	F0F1 ATP synthase epsilon subunit	NS	NS	**UP**
wOo_06870	6	0.002	Dihydrofolate reductase	NS	NS	**UP**
wOo_08030	6	0.002	Cytochrome c type biogenesis protein CcmE	NS	NS	**UP**
wOo_09930	6	0.004	Hypothetical protein	NS	NS	**UP**
wOo_06060	6	0.003	Hypothetical protein	NS	NS	**UP**
wOo_06810	5	0.003	Stress induced morphogen BolA	NS	NS	**UP**
wOo_03940	5	0.003	Asp tRNA Asn Glu tRNA Gln amidotransferase C subunit	NS	NS	**UP**
wOo_07890	5	0.008	Hypothetical protein	NS	NS	**UP**
wOo_06380	5	0.003	Uroporphyrinogen III synthase	NS	NS	**UP**
wOo_09480	5	0.007	Lipoprotein OsmY ortholog	NS	**UP**	NS
wOo_03690	5	0.006	tRNA Ala TGC	NS	NS	**UP**
wOo_01450	5	0.005	Hypothetical protein	NS	NS	**UP**
wOo_00340	5	0.007	NAD specific glutamate dehydrogenase	NS	NS	**UP**
wOo_10280	5	0.004	Preprotein translocase subunit SecY	NS	NS	**UP**
wOo_04360	5	0.008	Hypothetical protein	NS	NS	**UP**
wOo_03270	5	0.009	tRNA Val TAC	NS	**UP**	NS
wOo_02410	5	0.008	tRNA Pro TGG	NS	NS	**UP**
wOo_00740	5	0.008	Hypothetical protein	NS	NS	**UP**
wOo_06050	5	0.010	Deoxycytidine triphosphate deaminase	NS	NS	**UP**
wOo_01870	4	0.002	Peptidyl tRNA hydrolase	NS	**UP**	NS
wOo_03810	3	0.006	Ribonuclease HII	NS	NS	**UP**
wOo_03020	3	0.003	HesBYadRYfhF family protein	NS	**UP**	NS
wOo_04240	3	0.007	GTPase ObgE	NS	NS	**UP**
wOo_10010	3	0.006	tRNA processing exoribonuclease BN	NS	UP	NS
wOo_08780	-2	0.001	ATP-dependent exo DNase exonuclease V beta subunit RecB	NS	NS	**DOWN**
wOo_01620	-2	0.002	50S ribosomal protein_L10	NS	**DOWN**	NS
wOo_07060	-2	0.000	Transketolase	NS	NS	**DOWN**
wOo_09420	-3	0.001	Permease	NS	NS	**DOWN**
wOo_10150	-3	0.006	Hypothetical protein	NS	NS	**DOWN**
wOo_00160	-5	0.009	DnaK suppressor protein	**DOWN**	NS	NS
wOo_10680	-5	0.008	Ferredoxin	**DOWN**	NS	NS
wOo_10190	-6	0.001	Cytochrome C oxidase assembly protein	**DOWN**	NS	NS

Wolbachia gene transcript expressions upregulated (**UP**) or downregulated (**DOWN**) at weeks 8 (T8) or 52 (T52). NS no significant expression. → indicates direction of change.

Most of the *Wolbachia* DE transcripts at T52 were upregulated from values at both T8 and T4 as *Wolbachia* recovered from the antibiotic stress. At T8, rimM, a ribosomal

maturation protein, was upregulated but severely downregulated by ADT when compared to SLT. The highly regulated phosphatase may be involved in energy metabolism or detoxification, while rimM is an efficient processor of 16S rRNA (Bylund *et al.* 1998).

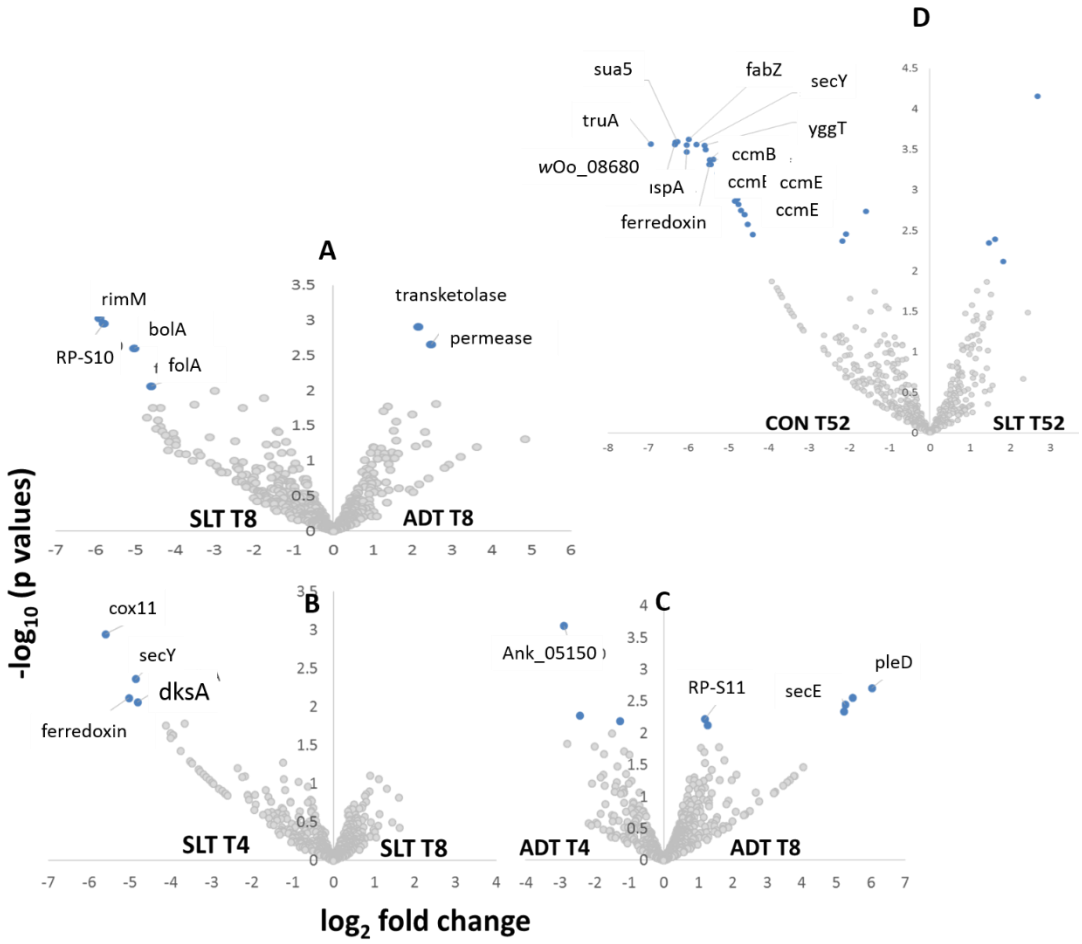

Figure 5.5: Volcano plot of time and group effects on *Wolbachia* expressed transcripts from bovine onchocercomata. Plots show contrast of DE transcript in the form of log-transformed fold changes and p values between (A) Sub lethal therapy (SLT) and Adulticidal therapy (ADT) at 8 weeks, (B) SLT at T4 and T8, (C) ADT at T4 and T8, and (D) untreated control and SLT at T52.

The response of *Wolbachia* to antibiotic treatments was assessed by comparing transcript numbers of the two treatment groups (Appendix 0). At T4, the earliest effect of ADT was the highly upregulated transcripts from response regulators and

secretion systems (pleD and secY) observed between ADT and SLT (Table 5.7). At T8, in addition to rimM, 30S ribosomal protein S10, folA and stress induced morphogen (BolA) gene were significantly less expressed in the ADT, while permease and transketolase showed greater expression than in the SLT-treated *Wolbachia* (Figure 5.5A). Although most transcripts of the SLT-treated *Wolbachia* were longitudinally upregulated at T52, they were still significantly inferior to the level of the untreated controls (Figure 5.5D). Hence, the *Wolbachia* had not fully recovered from treatment at T52.

Table 5.7: Macrofilaricidal effects on *Wolbachia O. ochengi* transcripts

Gene ID	log_2 FC	P-value	Gene Annotation	Differential Expression	
				T4 (CON→ADT)	ADT_T4→ADT_T8
wOo_10280	10	0.007	Preprotein translocase subunit SecY	**UP↑**	NS
wOo_06960	10	0.003	Response Regulator PleD	**UP↓**	**UP**
wOo_08250	9	0.009	Ribosome-binding factor A	**UP**	NS
wOo_08620	5	0.003	Hypothetical protein	NS	**UP**
wOo_01580	5	0.004	Preprotein translocase subunit SecE	NS	**UP**
wOo_05010	5	0.005	tRNA-Glu-TTC	NS	**UP↑**
wOo_02950	2	0.003	Geranylgeranyl pyrophosphate synthase	NS	**UP**
wOo_06350	1	0.007	WAS family protein	NS	**UP**
wOo_10250	1	0.006	30S ribosomal protein_S11	NS	**UP**
wOo_03610	-1	0.007	NADPH-dependent glutamate synthase beta chain	NS	**UP**
wOo_02790	-2	0.008	Type_IV secretory pathway VirB4 component	NS	**DOWN**
wOo_09850	-2	0.005	Glutamine amidotransferase domain-containing protein	NS	**DOWN**
wOo_05150	-3	0.000	Ankyrin repeat containing protein	NS	**DOWN**

↑ Also upregulated by SLT; ↓ downregulated by SLT at T52; **DOWN or UP** regulated: by ADT
NS = not significant differences

5.3.4.2 *Effects of ADT Treatment on Wolbachia Transcripts*

All DE transcripts after macrofilaricidal therapy observed at T4 and T8 are reported in Table 5.7. The most upregulated *Wolbachia* gene by ADT at T8, PleD (Figure 5.5, C),

was upregulated earlier at T4 in the ADT/CON (Table 5.7) or ADT/SLT (FC = 8, P = 0.003) comparisons. PleD is a cell cycle response regulator (Ontology: K02488) that initiates motility and cell density before cell division while under stress, could mutate to cause pleiotrophic polar defects during cell differentiation (Aldridge *et al.* 2003). The upregulation of pleD was accompanied by an increased regulation of ribosome biogenesis and protein translocation (SecY) and export across membranes (Ko03060).

Only three *Wolbachia* transcripts were downregulated by ADT (Table 5.7), although transcripts regulating glutamine amidotransferase domain containing protein and ankyrin repeat containing protein domains were not significantly different from expression in the CON. However, VirB4 (wOo_02790), a type IV secretion system protein (K03199), was the only transcript that was downregulated by ADT when compared to the CON (log FC = 2, P < 0.01).

5.4 Discussion

RNA-Seq of *O. ochengi* onchocercomata, the closest biological relative of *O volvulus* and arguably its best research system (Trees 1992), was used to evaluate changes in gene expression between the treated and untreated nodules for the first time. Prior to this study, we evaluated female worm proteomics under similar treatments (Chapter 4, results 4.3). Unlike with female worm proteomics, two-thirds of the quantified transcripts obtained from nodules were derived from the filarial parasite and the majority of those exhibiting significant changes were uncharacterized. As the nodule is characterised by an adult female worm circumscribed within collagenous tissues (Bwangamoi 1969; Hildebrandt *et al.* 2014), and Mf released in their hundreds every day, it was expected to express more worm transcripts than cattle-derived transcripts. The high expression of worm transcripts is an indication that the worm orchestrates control of its nodule environment. Post-genomic technology has been of great service to generate knowledge on the biology of filarial nematodes, leading to the identification of novel vaccine candidates, drug targets and biomarkers for diagnosis of *O. volvulus* in humans (Lustigman *et al.* 2017; Stoltzfus *et al.* 2017). However, a lot still needs to be done to characterize and identify the functions of these expressed worm genes and use this technology to elucidate the mechanism of action of drugs that are macrofilaricidal.

The first evidence of the efficacy of ADT in killing adult worms in this study was the low level of RNA quantified from ADT treated nodules at T52. The bovine tissue collected at the site of resorbed nodules were mostly fibrous connective tissue and

could not have been homogenized easily by the technique employed, as it did not include a pre-digestion step. However, we expected some level of scavenger immune cells to be present if the time of worm death was close to T52. This was not the case, implying that the worms died shortly after 36 weeks post-treatment and macrophages might have had enough time to phagocytose debris, leading to only a remnant of acellular scar tissue at the original nodule location. Eosinophil degranulation onto the worm's cuticle precedes worm death (Hansen *et al.* 2011), but the mechanism of worm killing caused by eosinophils has not been clearly elucidated. Population dynamic studies of immune cells that surround the worm after treatment did not illustrate significant increases in macrophages around the dying worm at any time points, in contrast to the major changes in neutrophil and eosinophil numbers (Nfon *et al.* 2006). Nevertheless, qualitative changes in alternatively-activated macrophage activity play a key role in the recruitment of helminthotoxic (CCR3-dependent) eosinophils in the *B. malayi* murine model (Turner *et al.* 2018). The first immune cells to be recruited at the site of infection are neutrophils (Delves *et al.* 2011). Neutrophils stimulated by WSP attract more neutrophils to be recruited to the site with little effect on worm viability. The withdrawal of WSP and the endosymbiotic bacteria may deprive worms of immune defence modulation and essential energy in the form of ATP, which is needed particularly during the reproductive phase of the worm (Darby *et al.* 2012). Perhaps to compensate for energy deficiency following depletion of *Wolbachia*, the filarial ATP synthase beta-subunit transcript was one of the most upregulated transcripts in the ADT group at T8.

Lack of reference transcriptomes of bovine eosinophils and neutrophils made the evaluation of the type of immune cells involved in worm death impossible without making assumptions. Histological studies have unequivocally shown that the depopulation of neutrophils around antibiotic-treated worms is preceded by sustained depletion of the endosymbiont *Wolbachia* from all tissues and that the presence of *Wolbachia* could not be detected by immunohistochemistry at T8 (Gilbert *et al.* 2005; Nfon *et al.* 2006). However, by RNA-Seq we observed that *Wolbachia* were not completely depleted at T8, and most of the *Wolbachia* transcripts were not downregulated, contrary to what would be expected from PCR analysis of doxycycline-treated human samples (Hoerauf *et al.* 2008b). All 640 *Wolbachia* transcripts were detected in the ADT treated nodules at T8, but very few of them had significant DE. The most upregulated transcript at T8 (PleD) is a response regulator (a member of two-component signal transducer system) that is known to regulate intracellular replication in relatives of *Wolbachia* such as *Ehrlichia chaffeensis* (Kumagai *et al.* 2011). PleD was also upregulated in the untreated control group at T52, perhaps due to ageing and natural death of one nodule in the cow ear-tagged 239. Stress-induced morphogen bolA gene, upregulated by SLT and downregulated by ADT regulates metabolic activities associated with resistance to antibiotic stress as simulated on the MetaCyc data modelling for metabolism and enzyme activity (Caspi *et al.* 2016). The role of the bolA transcript after SLT therapy may bring to light an important role in the cattle *O. ochengi* filarial model during validation trials of new or repurposed anti-*Wolbachia* drugs.

Primary components of protein translocation channels were upregulated. They are important exporters of unfolded proteins across membranes (KO03060). SecY and SecE are upstream to SecB and complexed with SecA to improve the efficiency of peripheral membrane ATPase (Flower 2007). Further complexing with SecD, SecF and YajC improve ATP binding (Flower 2007; Lycklama *et al.* 2013). SecY interacts with SecE to form the central channel of the preprotein translocase complex (Lycklama *et al.* 2013), thereby facilitating the release of transported proteins across membranes. However, during previous *in vitro* experiments of *Wolbachia* under doxycycline-induced stress, a different protein secretion system from the twin-arginine translocase was upregulated (Darby *et al.* 2014). The ribosome-binding factor A, an essential component for ribosome biogenesis, was probably upregulated in response to downregulation of the 30S ribosomal subunit.

Tetracyclines interact with ribosomes (Tritton 1977), interfering with their ability to coordinate protein synthesis (Chopra and Roberts 2001). Both ribosome maturation protein M (RimM) and 30S ribosomal protein transcripts were downregulated by ADT at T8 and T4 but following withdrawal of antibiotic effects in the SLT group, transcription of RimM and 30S ribosomal proteins increased to a peak at T52. RimM facilitates the maturation of the 30S ribosomal subunit of rRNA by increasing the efficiency of processing 16S rRNA to bind to ribosomal protein S19 (Bylund *et al.* 1998; Suzuki *et al.* 2007). The 30S ribosomal subunit ensures the efficiency of protein synthesis by coordinating the proper and accurate alignment of mRNA and tRNA for efficient genetic translation (Carter *et al.* 2000).

Previous analysis of *Wolbachia* gene expression to determine the symbiotic relationship with the host *O. ochengi* suggested that the endosymbiont supplies energy to and enhances immune protection of the worm (Darby *et al.* 2012). Both adulticidal and sublethal oxytetracycline therapy upregulated worm HSP60, which is often expressed under stressful conditions to illicit immune responses against diseases by acting as danger signal (Grundtman *et al.* 2011). Assuming that processes in *O. ochengi* and *C .elegans* are similar, cell death in neurons was initiated by the upregulation of CES-1 and CES-2 (Ellis and Horvitz 1991; Wang *et al.* 2006). These CES transcripts were downregulated in the untreated control and SLT groups from T4. However, the trigger for generalized cell death is controlled by the CED-3 and CED-4 genes (Wang *et al.* 2006), which as at T8 in the ADT group were not expressed. Thus, although the precise links between the various transcripts are unclear, worm death seemed to be associated with targeted cell death in neurons alongside upregulation of the stress protein HSP 60 and the innate immune receptor MARCO. This receptor is a class A scavenger molecule expressed on macrophages (Kraal *et al.* 2000), but is also conserved in *C. elegans*. It enhances carbon nanotube uptake (Hirano *et al.* 2012) and phagocytic cell mediated immunity (Zhang *et al.* 2018). Its upregulation within worms at T8 by macrofilaricidal therapy relative to ineffective therapy was accompanied by uncharacterised transcripts, antigen maltose binding protein, prolyl 4-hydroxylase, and cre-tag-297 protein. It would be exciting to find out the role of these immune responses within the worm and whether they are also involved in regulating *Wolbachia* density under normal conditions.

The highest number of regulated worm gene transcripts were from the untreated control worms with significant enrichments in homeobox domain and collagen helix protein coding genes which play important roles in tissue repair, routine maintenance of growth and reproductive development (Kachgal *et al.* 2012). This was to be expected during a year-long experiment, as there is seasonal variation in the reproductive pattern of filarial parasites (Achukwi *et al.* 2000) which would have led to significant changes in the CON transcription. However, treatment specifically regulated worm cuticle transcripts such as cuticle collagens, prolyl 4-hydroxylases and a number of others from both by the SLT and ADT groups, which serves as indirect evidence of eosinophil degranulation on the worm as previously suggested (Hansen *et al.* 2011). There was sustained transcription of extracellular matrix protein and DNA repair genes; *i.e.*, associated with HAPLN1 involvement in cartilage repair (Franzén *et al.* 1981; Hering *et al.* 1995) and histone H2A type 1 gene in DNA repair[10].

The SLT accounted for the largest transcription changes from the bovine host, which provides fibro-collagenous tissue that encapsulates and entangles the female worm (Bwangamoi 1969), blood vessels and access to nutrition needed for energy, growth and respiration by the worm (George *et al.* 1985; Smith *et al.* 1988), and neutrophils that infiltrate into the capsular space and may be responsible for the worm's longevity (Gilbert *et al.* 2005; Nfon *et al.* 2006; Hansen *et al.* 2011). These huge

[10] http://www.uniprot.org/uniprot/P0C0S9

changes in bovine transcripts both from the SLT and ADT treatments, buttress the fact that the worm's environment plays an important role in its survival.

Changes in immune responses associated with glycoside hydrolase family 22 proteins were significant at T8. Downregulation of leukotriene B4 receptor 2 (LTB4R2) transcript variant X1 found on B-lymphocytes may depict inhibition of proinflammation and reduction of recruitment of monocytes, neutrophils and eosinophils (Crooks and Stockley 1998). Solute carrier family 5 member 7 is a macrophage protein (SLC5A7). Another common inhibitor of cytotoxicity by immune cells is the increased production of ligands that blocks cytotoxic receptor sites on macrophages and NK cells. Transcripts for NKG2D ligand 4 were upregulated while histone H2A type 1 and joining chain of multimeric IgA and IgM were downregulated by SLT at T8. Damaged or infected cells usually express non-self-proteins that are identified as danger signals (Delves *et al.* 2011). These antigenic proteins constitute ligands that are identified by scavenger immune cells and NK with NKG2D receptors. Natural cytotoxicity receptors (NCR) are found on NK cells, macrophages, and lymphocytes and when activated initiate the primary immune reaction to pathogens (Haik *et al.* 2016). The upregulation of NKG2D ligand 4 transcripts is key evidence of circulation of immunomodulatory molecules that block NCRs and probably promote immunomodulation against worms exposed to SLT. However, NKG2D ligand 4 transcription was downregulated by ADT, thereby perhaps enabling NK and other mononuclear immune cells to elicit proinflammatory processes. Soluble ligands generated under heat stress conditions such as burns, often block NKG2D receptor sites, preventing cytotoxic activation by immune cells and diminishing the ability of

these immune cells to control new infections (Haik *et al.* 2016). This mechanism is currently being exploited and the NKG2D receptors are a therapeutic target in the control of cancer (Spear *et al.* 2013). Interestingly, NK cells have been shown to secrete IL-4 and IL-5, as well as upregulate expression of natural cytotoxicity receptors, when exposed to live *B. malayi* Mf.

Transcriptional differences were observed between ADT and SLT at T4, even though they had received the same dose of oxytetracycline treatment by that point. Several groups of transcripts were upregulated from *Wolbachia*, the worm and the bovine host in the ADT group relative to the SLT group. Evaluation of worm fecundity and Mf load at T0 (pre-treatment) indicated that ADT treated animals were more fertile and produced higher Mf loads. If *Wolbachia* is the power house of the worm as we earlier hypothesized (Darby *et al.* 2012), then it is likely that worms treated at peak reproduction would respond differently to antibiotic treatment than others. With an additional intramuscular dose of oxytetracycline at T4, the main effect of ADT was visible with the upregulation of bovine VDAC1, a mitochondrial outer membrane channel protein through which ATP is exported out of mitochondria (Choudhary *et al.* 2014). In the eukaryotic cell outer membrane, it also regulates cell volume. VDAC1 is involved in the apoptosis pathway and is often considered as a biomarker for mitochondrial dysfunction (Palmeira *et al.* 2014). Multidrug resistance-associated proteins 4 (Zimin *et al.* 2009), which are ATP-binding cassette transporters proteins (Dai *et al.* 2017), were significantly highly expressed in the ADT-treated nodules, alongside histone H2A type 1 and ATP synthase β-subunit. These changes could reflect a direct effect of the antibiotic on worm mitochondrial translation (Moullan *et al.* 2015) or

the worm's compensatory mechanisms for the energy gap created by *Wolbachia* depletion during antibiotic therapy (Darby *et al.* 2014).

5.5 Conclusions

Adult filarial worms were killed before week 52 after treatment. Nodule remnants at T52 processed for RNA extraction lacked enough mRNA for sequencing. It can be concluded that changes observed from samples at weeks four and eight of this study were early indicators of worm death. Hence, upregulation of transcripts that regulate apoptosis such as cell death specification protein 2 and ATP synthase beta subunit amongst others, reflected changes that either contributed to worm death or were failed attempts to mitigate the ADT regimen. *Wolbachia* was not killed by the ADT dose at T8, but upregulated genes involved in stress responses and energy metabolism. In the SLT group, *Wolbachia* and its worm host may have survived due to upregulation of symbiont energy metabolism during weeks four through eight, although by the end of the experiment, *Wolbachia* energy metabolism was severely downregulated in this group. Bovine transcripts regulated in the SLT favoured tissue homeostasis and angiogenesis. There is a need for further studies to be conducted on ADT nodules to include time-points closer to the time of worm death after the last treatment at T36.

Chapter 6 General Discussion, Conclusions, and Recommendations

6.1 General Discussion

The search for macrofilaricidal drugs is a major contribution in achieving the objectives of global eradication of onchocerciasis, given the imminent risk of resistance to IVM (Osei-Atweneboana *et al.* 2011; Nana-Djeunga *et al.* 2014; Doyle *et al.* 2017). This study evaluated the immunochemotherapeutic efficacy of mutated onchocystatin (Chapter 3, section 3.2.5), an immunogenic protein found on the adult worm's cuticle and secreted by all its developmental stages except mature Mf (Lustigman *et al.* 1991). We then attempted to identify the mechanisms that lead to eosinophil degranulation onto the worm cuticle using the *O. ochengi* research model for the first time. We tested the possibility of shortening the duration of antibiotic therapy by prior vaccination of infected cattle. In natural infection, some aged infections tend to be self-limiting, while other exposed persons or animals resist infection and are considered as putative immunes, PIs (Ward *et al.* 1988; Boyer *et al.* 1991; Elson *et al.* 1995). The serum of PIs contain cytophilic antibodies (IgG3) that targeted onchocystatin (Chapter 1, Section 1.2.1) and so we hypothesized that immunotherapy would strengthen Th2-mediated effector mechanisms, principally eosinophils, against the adult worms (Hansen *et al.* 2011). Unfortunately, our results (Chapter 3, Section 3.3.3) rejected this hypothesis. The main parameter used in evaluating worm death was motility of adult worms (score of zero = death) and resolution of nodules containing dying worms (Langworthy *et al.* 2000; Bronsvoort *et al.* 2005). There is a small risk that the method used might have included false

negatives (i.e., “terminally ill” worms that would have died shortly after the experiment terminated), as there is not yet a mechanism for evaluating dying adult worms with certainty. In the gold standard ADT protocol, worm death is confirmed by the resorption of nodules or the presence of degenerated, fragmented and calcifying worm tissues with more than a 60% reduction in worm viability (Gilbert *et al.* 2005). The calculation of worm viability factored natural changes using the untreated control. We assumed at the start of the experiment that no cattle recruited for the experiment purchased from the market within four weeks from the start of the study had been prior treated with oxytetracycline. However, after trypanocides and penicillin-based antibiotics, oxytetracycline is the third most used self-medication by cattle farmers in the Adamawa Region of Cameroon (personal observation). Thus, some effects on worm viability before the experiment began are difficult to exclude.

In humans treated with doxycycline for four or six weeks, significant macrofilaricidal effects were observed only at 20 - 27 months after treatment (Hoerauf *et al.* 2008b). However, evidence of infertility (female worm sterilisation) appeared at 6 months post-treatment. Previous studies might have erred in making conclusions from observations collected within 18 months of sampling; that is, macrofilaricidal activity was not initially confirmed (Hoerauf *et al.* 2003; Supali *et al.* 2008). In this immunochemotherapy trial, there were no obvious indicators that extending the period of observation would have altered the results reported. Notwithstanding, both vaccinations alone or in combination (immunochemotherapy) elicited a significant systemic immune response against onchocystatin and local eosinophil

recruitment into the nodules, with the immunochemotherapeutic group displaying the highest median eosinophil count at T12. This finding validated the quality of the antigen used (Chapter 3, Section 3.2.5) in this vaccination trial and suggests that other immunomodulatory mechanisms exist to compensate for the targeting of one pathway.

To further filarial vaccine research, it is vital to identify the best vaccine targets capable of preventing infection or those that can cure and prevent reinfection. The focus has been on worm secretary/excretory products or surface antigens, with lead vaccine candidates being Ov-103 and Ov-RAL-2, which are expressed in all lifecycle stages (Hess *et al.* 2014; Hotez *et al.* 2015; Hess *et al.* 2016; Lustigman *et al.* 2018). But the establishment of infection and worm survival, reproduction and viability depends on *Wolbachia* surface proteins both within, and as effectors outside (Melnikow *et al.* 2011; Armstrong *et al.* 2014), the worm that activate pro-inflammatory immune responses via interactions with TLR-2/4 (Brattig *et al.* 2004; Gillette-Ferguson *et al.* 2007), which may protect the worm and inhibit apoptosis of neutrophils (Bazzocchi *et al.* 2007). The interaction of *Wolbachia* PAL with TLR 2/6 induces Th1 immune responses against Mf and causes skin and ocular immunopathogenesis (Turner *et al.* 2009; Tamarozzi *et al.* 2011).

Recently it was also shown that *Wolbachia* muramyl dipeptide interacts with NOD2 receptors of keratinocytes to induce pro-inflammatory neutrophil responses that limits the establishment of infective larvae within the skin (Ajendra *et al.* 2016). Although *Wolbachia* has no organised cell wall, it can synthesises muramyl dipeptide, a peptidoglycan component of purported bacterial cell wall that binds intra-

cytoplasmic NLR protein NOD2 (Grimes *et al.* 2012) found in keratinocytes, neutrophils, APC, monocytes and macrophages. The role of the NOD2 receptor protein in the immune regulation of filarial disease has been investigated only in the *L. sigmondontes* murine model of human lymphatic filarial using neutropenic mice (Pionnier *et al.* 2016) and NOD2-depleted mice (Ajendra *et al.* 2016). The activation of NOD2 receptors triggers NF_kB-induced upregulation of pro-inflammatory genes, neutrophil infiltration and activation via protein kinase RIP2 (Hasegawa *et al.* 2008) and transforming growth factor-activated kinase 1 (TAK1) signalling pathways in epidermal cells (Kim *et al.* 2008). The trigger of NF_kB via TAK1 signalling plays a homeostatic role in the regulation of apoptosis (Pionnier *et al.* 2016). This mechanism could be exploited in future research to enhance efficacy of vaccine trials in a manner yet to be determined. Although the efficacy of a vaccine against a *Wolbachia* antigen could be limited by the primary intracellular location of the symbionts, early removal of *Wolbachia* excretory/secretory products might inhibit the establishment of infective larvae and expose filariae to enhanced Th2-mediated effector mechanisms. Following a previous attempt to vaccinate against WSP in the *L. sigmodontis* model, which led to increased worm burdens if the vaccine was delivered in Freund's adjuvant, but not alum (Lamb *et al.* 2008), it is clear that such an approach would need to carefully optimise the direction of the immune response in the early phase after vaccination. Since *Wolbachia* also has a key role in facilitating the migration of infective larvae via TLR2-mediated stimulation of histamine release from mast cells, promoting vascular permeability (Specht *et al.* 2011), the potential benefits of targeting *Wolbachia* in a prophylactic strategy should be a focus of future research.

Full validation of the transcriptomic and proteomic results reported herein would be very challenging. Even for the bovine host, relatively few antibodies that could be used in immunohistochemistry to identify the cellular sources of immune mediators and effectors are commercially available. The production of custom antibodies can be time-consuming and expensive, and many antibodies fail to recognise their targets in fixed tissue sections. *In-situ* hybridization to localise mRNA transcripts with specific nucleic acid probes might be a more practicable approach, but would leave open the question of how to link mRNA and protein expression throughout the nodule and would not be able address changes in preformed or secreted proteins, such as neutrophil and eosinophil granule proteins (Cowland Jack and Borregaard 1999). Thus, when probing the mechanisms of worm death or survival after antibiotic therapy from the female worm proteome and onchocercoma transcriptome, a new analytical tool may be needed to draw any meaningful conclusion with respect to the specific source of the regulated proteins and transcripts.

The new framework for correlating transcription and protein abundance proposed by Morimoto and Yahara (2018) could be used to further analyse existing datasets to identify stress responses from the antibiotic treatments. The vaccinated groups were excluded from transcriptomic and proteomic studies because of a lack of significant effect on worm viability. We used the female worm proteome to compares changes at T0, T12 and T36 and the nodule transcriptome from alternate time points (T4, T8 and T52) due to problems with RNA quality in several nodule extracts. Nonetheless, we would expect transcript and protein-level changes to show similar effects, as the latter, while separated by a variable time-lag, should be preceded by the former. The

effects of transcriptional changes that will result in worm death might persist over a long period, so that observations of the onchocercomata transcriptome at T4 and T8 were expected to be valuable in explaining proteomic data at T12 and T36. However, we would have preferred to run proteomic and transcriptomic analyses from the same time-points had we not lost equivalent samples during total RNA extraction using the RNAqueous Total RNA Isolation Kit from Ambion. The major limitation of this kit was the frequent blockage of the filter as nodule homogenates were forced through with a syringe. Secondly, RNA quality declined after DNA removal from extracted RNA was done before final purification and submission for sequencing. We resorted to using the available alternate nodule samples with QIAGEN® RNeasy MIDI or MAXI kits, which gave excellent results. Homogenisation, RNA extraction and purification were carried out the same day in Cameroon. The main advantage of the QIAGEN® RNeasy MIDI kits was the one-step approach, where all procedures up to DNA removal were performed at room temperature before eluting purified RNA for storage at -80°C. The procedure was more appropriate for the extraction of large quantities of total RNA from nodules compared with the Ambion RNAqueous kits.

Proteomic and transcriptomic data from the ADT group supported previous findings that oxytetracycline has a bacteriostatic effect on *Wolbachia* and interferes with ribosomal activity and protein synthesis (Chopra *et al.* 1992; Connell *et al.* 2003). Although we could not correlate changes in the endobacteria with those of the worm, it was evident that key processes that resulted in worm death were activated.

In this study, it is worth re-emphasizing the surprising extensive expression of *Wolbachia* genes at eight weeks (Chapter 5, Section 5.3.4.2) and a few proteins thirty-

six weeks (Chapter 4, Section 4.3.2.2) after start of oxytetracycline treatment when we least expected them given previous allusions that their complete depletion led to worm death based on histological observation (Hoerauf *et al.* 2001; Hoerauf *et al.* 2003; Gilbert *et al.* 2005; Hoerauf *et al.* 2008b; Frederic Landmann *et al.* 2011). This brings to light a major limitation of some of the research tool used so far especially when interpretation is made without proper knowledge of the biology of an organism. The effect of ADT on the expression of pleD transcript (Aldridge *et al.* 2003) and its contribution to positioning *Wolbachia* where it can be easily identified by immunohistochemistry techniques will need to be investigated in future studies. The identification of the role of *Wolbachia* effectors (Rice *et al.* 2017) and effects on secretory pattern could contribute in explaining whether changes observed by immunohistochemistry were due to relocation of WSP or not.

6.2 General Conclusions

6.2.1 Control of Onchocerciasis by Vaccination

This study confirms the potency and immunogenicity of mutated *O. ochengi* onchocystatin as a vaccine candidate. The enhanced immune response observed from the vaccinated *O. ochengi* infected cattle is evident by the significantly raised and sustained IgG levels over four weeks. The onchocystatin, as expected, did attract the highest number of eosinophils into the nodules of the immunochemotherapy group during the first 12 weeks, but eosinophil levels dropped thereafter; whereas those in the ADT group continued to rise, thus effecting worm killing. It can be concluded that the vaccination failed due to a lack of booster dose after treatment, which might have sustained a high eosinophil level between 12 and 36 weeks, considered the critical period for worm killing.

6.2.2 Worm Proteomics and Transcriptomics

Our female worm proteomic data confirmed previous findings that worm death is preceded by depletion of *Wolbachia* and neutrophils, while eosinophil degranulation is enhanced. All proteins of *Wolbachia* and bovine origin involved in proinflammatory responses were downregulated. The bovine response was dominated by neutrophil granule proteins. Meanwhile, the majority of those few worm proteins exhibiting regulation were upregulated. Eosinophil granule proteins were also upregulated, and their effects on the worm could be deduced from the upregulation of cuticle and collagen proteins. Transcriptomic data did not significantly amplify our understanding of the immune mechanisms leading to worm killing. Mitochondrial transcripts and those regulating cuticle formations of treated worms were upregulated, while

Wolbachia transcripts were downregulated. Granulocytes within the nodules are rich in preformed granules and contain relatively little RNA (~1 µg per 10^7 cells in humans) (Tamassia *et al.* 2014). It was not possible to determine with precision the regulatory effect of antibiotic therapy on this cell population. However, the worms (the organism from which the highest number of transcripts could be quantified in the onchocercoma) showed evidence of interference with ingestion and apoptotic changes as early as eight weeks after the start of protracted treatment. Transcriptomic data enabled us to observe changes during ADT therapy that eventually killed adult worms, leading to resorption of several nodules by week 52.

6.3 Recommendations

6.3.1 Immunochemotherapy

1. The vaccination trial using mutated onchocystatin for immunochemotherapy should be repeated, with the cattle given booster doses at 4 and 8 weeks after treatment in the expectation of sustaining nodule eosinophilia for a longer period. Ideally, sampling and evaluation of animals for worm viability should last for a duration of two years.
2. In addition, the therapeutic effect of onchocystatin on IVM-attenuated worms should be evaluated, with monitoring of effects on both adult worm viability and skin Mf.

6.3.2 Research Cattle and Sampling

The quality and availability of *O. ochengi*-infected experimental cattle has always been a challenge. In addition, the clinical history of cattle, especially past treatments with oxytetracycline, is not always available. Even though we made every effort to obtain healthy cattle, it is recommended that the research group should own a farm for the production *O. ochengi*- infected cattle along the River Vina. In the absence of this farm, future purchases of infected experimental cattle should take place at least six months before the start of experiments.

The mechanism of evaluating male worm viability needs to be standardized to enable their absence to be factored alongside that of females. We recommend a specific study that will not only determine the normal range of male worms per female in nodules, but also solve the equation on how to factor viability of dying worms

following treatment in forecasting eventual worm death with high degree of accuracy.

The strong positive association between observed nodule diameter, Mf densities, fecundity, and the number of viable worms observed only in the ADT group indicates that the parameters measured and analysed were true estimates of viability. Future studies should model the contribution of each measurement of viability, although larger datasets will be needed, and parameters monitored for a longer time to validate complete worm mortality in the macrofilaricidal group.

The "gold standard" oxytetracycline therapy has rarely attained 80% macrofilaricidal efficacy. In the current study, worms with high fecundity tended to respond to initial antibiotic therapy differently from those with low fecundity. Future studies should verify this hypothesis, as it might have serious implications for the application of antibiotic therapy for human onchocerciasis patients in future.

6.3.3 Worm Proteomics and Transcriptomics

There remain a considerable number of uncharacterised nematode genes. In the current study, many of these genes were affected by antibiotic therapy. While several groups worldwide continue to annotate filarial genomes and integrate data on WormBase ParaSite (Howe *et al.* 2017), functional characterisation of the thousands of unique genes in filarial nematodes is a massive undertaking that requires significant investment.

As leukocytes and other immune-related cells often produce overlapping proteins and transcripts, the identification of the source of an important protein can be

difficult unless immunohistochemical methods are properly optimised. We recommend that research be intensified in characterising bovine immune cells with the goal of identifying unique markers for bovine neutrophils, eosinophils, and macrophages, as well as extending the range of available markers for lymphocyte subsets.

Given the presence of *Wolbachia* transcript in dying worms, studies will need to be conducted to re-evaluate the role of *Wolbachia* in worm killing. The effect of ADT on the expression of pleD transcript (Aldridge *et al.* 2003) and its contribution to positioning *Wolbachia* where it can be easily identified by immunohistochemistry techniques will need to be investigated. The identification of the role of *Wolbachia* effectors (Rice *et al.* 2017) and effects on secretory pattern could contribute to explaining whether changes observed by immunohistochemistry were due to relocation of WSP or not.

Appendix

Appendix 6-1: Regulated *Wolbachia* Transcripts. I. Longitudinal significant expressions

GENE ID	log_2 FC	P-value	Annotation	Comparison
wOo_06960	6	0.002	Response regulator PleD	ADT_T4vsT8
wOo_08620	5	0.003	Hypothetical protein	ADT_T4vsT8
wOo_01580	5	0.004	Preprotein translocase subunit SecE	ADT_T4vsT8
wOo_05010	5	0.005	tRNA-Glu-TTC	ADT_T4vsT8
wOo_06350	1	0.007	WAS family protein	ADT_T4vsT8
wOo_10250	1	0.006	30S ribosomal protein S11	ADT_T4vsT8
wOo_03610	-1	0.007	NADPH-dependent glutamate synthase beta chain	ADT_T4vsT8
wOo_09850	-2	0.005	Glutamine amidotransferase domain-containing protein	ADT_T4vsT8
wOo_05150	-3	0.000	Ankyrin repeat-containing protein	ADT_T4vsT8
wOo_00530	5	0.001	Hypothetical protein	CON_T4vsT52
wOo_03610	-2	0.009	NADPH-dependent glutamate synthase beta chain	CON_T4vsT52
wOo_03760	6	0.003	tRNA pseudouridine synthase A	CON_T4vsT8
wOo_06590	5	0.002	Preprotein translocase subunit SecD	CON_T4vsT8
wOo_07930	-2	0.009	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	CON_T4vsT8
wOo_08690	-2	0.001	Hypothetical protein	CON_T4vsT8
wOo_00530	5	0.001	Hypothetical protein	CON_T8vsT52
wOo_03610	-2	0.009	NADPH-dependent glutamate synthase beta chain	CON_T8vsT52
wOo_05670	7	0.000	Phosphatase	SLT_T4vsT52
wOo_08750	7	0.000	ABC-type Mn2+Zn2+ transport system permease component	SLT_T4vsT52
wOo_10500	7	0.000	30S ribosomal protein S10	SLT_T4vsT52
wOo_03860	6	0.000	Putative translation factor SUA5	SLT_T4vsT52
wOo_00220	6	0.000	tRNA-Ser-GCT	SLT_T4vsT52
wOo_07890	6	0.003	Hypothetical protein	SLT_T4vsT52
wOo_05560	6	0.002	RimM protein required for 16S rRNA processing	SLT_T4vsT52
wOo_01370	5	0.004	N6-adenine-specific methylase	SLT_T4vsT52
wOo_06810	5	0.003	stress-induced morphogen BolA	SLT_T4vsT52
wOo_06060	5	0.005	Hypothetical protein	SLT_T4vsT52
wOo_09480	5	0.007	Lipoprotein OsmY ortholog	SLT_T4vsT52
wOo_06870	5	0.006	Dihydrofolate reductase	SLT_T4vsT52
wOo_09200	5	0.008	NADH ubiquinone oxidoreductase 18 kDa subunit	SLT_T4vsT52
wOo_03940	5	0.007	Asp-tRNAAsnGlu-tRNAGln amidotransferase C subunit	SLT_T4vsT52
wOo_03270	5	0.009	tRNA-Val-TAC	SLT_T4vsT52
wOo_06050	5	0.010	Deoxycytidine triphosphate deaminase	SLT_T4vsT52
wOo_01870	4	0.002	peptidyl-tRNA hydrolase	SLT_T4vsT52
wOo_10010	3	0.003	tRNA processing exoribonuclease BN	SLT_T4vsT52
wOo_03020	3	0.004	HesBYadRYfhF family protein	SLT_T4vsT52

wOo_10260	2	0.007	30S ribosomal protein S13	SLT_T4vsT52
wOo_04550	2	0.007	bifunctional GMP synthaseglutamine amidotransferase protein	SLT_T4vsT52
wOo_06300	1	0.005	membrane protease subunit stomatinprohibitin-like protein	SLT_T4vsT52
wOo_07060	-1	0.010	transketolase	SLT_T4vsT52
wOo_04090	-1	0.006	inorganic polyphosphateATP-NAD kinase	SLT_T4vsT52
wOo_08840	-2	0.002	major facilitator superfamily permease	SLT_T4vsT52
wOo_09420	-2	0.007	permease	SLT_T4vsT52
wOo_01620	-2	0.002	50S ribosomal protein L10	SLT_T4vsT52
wOo_10190	-6	0.001	cytochrome C oxidase assembly protein	SLT_T4vsT52
wOo_00160	-5	0.009	DnaK suppressor protein	SLT_T4vsT8
wOo 10280	-5	0.004	preprotein translocase subunit SecY	SLT_T4vsT8
wOo_10680	-5	0.008	ferredoxin	SLT_T4vsT8
wOo_10190	-6	0.001	cytochrome C oxidase assembly protein	SLT_T4vsT8
wOo_10500	7	0.000	30S ribosomal protein S10	SLT_T8VsT52
wOo_06810	6	0.001	stress-induced morphogen BolA	SLT_T8VsT52
wOo_08750	6	0.000	ABC-type Mn2+Zn2+ transport system permease component	SLT_T8VsT52
wOo_03860	6	0.000	putative translation factor SUA5	SLT_T8VsT52
wOo_02750	6	0.001	cytochrome B561	SLT_T8VsT52
wOo_05670	6	0.000	phosphatase	SLT_T8VsT52
wOo_09200	6	0.002	NADH ubiquinone oxidoreductase 18 kDa subunit	SLT_T8VsT52
wOo_01370	6	0.003	N6-adenine-specific methylase	SLT_T8VsT52
wOo_05560	6	0.003	RimM protein required for 16S rRNA processing	SLT_T8VsT52
wOo_00840	6	0.006	F0F1 ATP synthase epsilon subunit	SLT_T8VsT52
wOo_06870	6	0.002	dihydrofolate reductase	SLT_T8VsT52
wOo_08030	6	0.002	cytochrome c-type biogenesis protein CcmE	SLT_T8VsT52
wOo_09930	6	0.004	hypothetical protein	SLT_T8VsT52
wOo_06060	6	0.003	hypothetical protein	SLT_T8VsT52
wOo_00220	5	0.001	tRNA-Ser-GCT	SLT_T8VsT52
wOo_03940	5	0.003	Asp-tRNAAsnGlu-tRNAGln amidotransferase C subunit	SLT_T8VsT52
wOo_06050	5	0.003	deoxycytidine triphosphate deaminase	SLT_T8VsT52
wOo_07890	5	0.008	hypothetical protein	SLT_T8VsT52
wOo_06380	5	0.003	uroporphyrinogen-III synthase	SLT_T8VsT52
wOo_09480	5	0.007	lipoprotein OsmY ortholog	SLT_T8VsT52
wOo_03690	5	0.006	tRNA-Ala-TGC	SLT_T8VsT52
wOo_01450	5	0.005	hypothetical protein	SLT_T8VsT52
wOo_00340	5	0.007	NAD-specific glutamate dehydrogenase	SLT_T8VsT52
wOo_10280	5	0.004	preprotein translocase subunit SecY	SLT_T8VsT52
wOo_03270	5	0.007	tRNA-Val-TAC	SLT_T8VsT52
wOo_05010	5	0.009	tRNA-Glu-TTC	SLT_T8VsT52
wOo_04360	5	0.008	hypothetical protein	SLT_T8VsT52
wOo_02410	5	0.008	tRNA-Pro-TGG	SLT_T8VsT52
wOo_00740	5	0.008	hypothetical protein	SLT_T8VsT52
wOo_03810	3	0.006	ribonuclease HII	SLT_T8VsT52

wOo_03020	3	0.003	HesBYadRYfhF family protein	SLT_T8VsT52
wOo_04240	3	0.007	GTPase ObgE	SLT_T8VsT52
wOo_10010	3	0.006	tRNA processing exoribonuclease BN	SLT_T8VsT52
wOo_10300	2	0.005	preprotein translocase subunit SecY	SLT_T8VsT52
wOo_04550	2	0.002	bifunctional GMP synthaseglutamine amidotransferase protein	SLT_T8VsT52
wOo_05000	2	0.008	Asp-tRNAAsnGlu-tRNAGln amidotransferase A subunit	SLT_T8VsT52
wOo_02550	1	0.004	ribonucleotide-diphosphate reductase subunit beta	SLT_T8VsT52
wOo_05050	1	0.007	fructose-bisphosphate aldolase	SLT_T8VsT52
wOo_03400	1	0.008	Kef-type K+ transport system membrane component	SLT_T8VsT52
wOo_01630	-1	0.006	50S ribosomal protein L7L12	SLT_T8VsT52
wOo_03330	-1	0.007	DNA gyrase subunit A	SLT_T8VsT52
wOo_04090	-1	0.004	inorganic polyphosphateATP-NAD kinase	SLT T8VsT52
wOo_06150	-2	0.008	DNA_recombination protein rmuC-like protein	SLT_T8VsT52
wOo_09720	-2	0.009	tRNA nucleotidyltransferasepolyA- polymerase	SLT_T8VsT52
wOo_09850	-2	0.001	glutamine amidotransferase domain-containing protein	SLT_T8VsT52
wOo_08840	-2	0.001	major facilitator superfamily permease	SLT_T8VsT52
wOo_07060	-2	0.000	transketolase	SLT_T8VsT52
wOo_08780	-2	0.001	ATP-dependent exoDNase exonuclease V- beta subunit RecB	SLT_T8VsT52
wOo_08240	-2	0.005	prenyltransferase	SLT_T8VsT52
wOo_09420	-3	0.001	permease	SLT_T8VsT52
wOo_10150	-3	0.006	hypothetical_protein	SLT_T8VsT52

Appendix 6-2. Regulated *Wolbachia* Transcripts. II. Between treatment significant expressions

GENE ID	Log_2 FC	P-value	Annotation	Comparison
wOo_10150	3	0.000	hypothetical protein	T52_CON_SLT
wOo_09720	2	0.008	tRNA nucleotidyltransferasepolyA- polymerase	T52_CON_SLT
wOo_02790	-2	0.008	Type_IV_secretory_pathway_VirB4_component	T8_CON_ADT
wOo_07400	2	0.004	histidyl-tRNA_synthetase	T52_CON_SLT
wOo_08690	1	0.004	hypothetical_protein	T52_CON_SLT
wOo_00040	-2	0.002	dihydrolipoamide_dehydrogenase_E3_component	T52_CON_SLT
wOo_07910	-2	0.004	HesBYadRYfhF_family_protein	T52_CON_SLT
wOo_07390	-2	0.004	NifU_family_protein	T52_CON_SLT
wOo_02160	-4	0.004	tRNA-Thr-GGT	T52_CON_SLT
wOo_06950	-5	0.003	response_regulator_PleD	T52_CON_SLT
wOo_10500	5	0.002	30S_ribosomal_protein_S10	T8_CON_SLT
wOo_09420	2	0.002	permease	T8_SLT_ADT
wOo_06810	5	0.001	stress-induced_morphogen_BolA	T8_CON_SLT
wOo_07060	2	0.001	transketolase	T8_SLT_ADT
wOo_06870	-5	0.009	dihydrofolate_reductase	T8_SLT_ADT
wOo_08250	9	0.009	ribosome-binding_factor_A	T4_CON_ADT
wOo_06960	10	0.003	response_regulator_PleD	T4_CON_ADT
wOo_06960	8	0.008	Response regulator PleD	T4_SLT_ADT
wOo_05890	-5	0.002	Ancestral ankyrin repeat-containing protein orthologue	T52_CON_SLT
wOo_02410	-5	0.002	tRNA-Pro-TGG	T52_CON_SLT
wOo_03370	-5	0.002	tRNA-Ser-TGA	T52_CON_SLT
wOo_06870	5	0.003	dihydrofolate_reductase	T8_CON_SLT
wOo_10280	10	0.007	preprotein_translocase_subunit_SecY	T4_CON_ADT
wOo_03270	-5	0.001	tRNA-Val-TAC	T52_CON_SLT
wOo_09200	5	0.008	NADH_ubiquinone_oxidoreductase_18_kDa_subunit	T8_CON_SLT
wOo_07020	4	0.004	hypothetical_protein	T8_CON_SLT
wOo_08060	3	0.006	NADH_ubiquinone_oxidoreductase_chain_N	T8_CON_SLT
wOo_03950	2	0.002	2-methylthioadenine_synthetase	T8_CON_SLT
wOo_05000	2	0.001	Asp-tRNAAsnGlu-tRNAGln_amidotransferase_A_subunit	T8_CON_SLT
wOo_06810	-5	0.002	stress-induced_morphogen_BolA	T8_SLT_ADT
wOo_04360	-5	0.001	hypothetical_protein	T52_CON_SLT
wOo_10300	2	0.006	preprotein_translocase_subunit_SecY	T8_CON_SLT
wOo_02950	2	0.009	geranylgeranyl_pyrophosphate_synthase	T8_CON_SLT
wOo_08760	-5	0.001	hypothetical_protein	T52_CON_SLT
wOo_00590	-5	0.001	hypothetical_protein	T52_CON_SLT
wOo_08690	2	0.005	hypothetical_protein	T4_CON_SLT
wOo_01780	-5	0.001	putative_monovalent_cationH+_antiporter_subunit_G	T52_CON_SLT
wOo_06380	-5	0.001	uroporphyrinogen-III_synthase	T52_CON_SLT
wOo_09590	-5	0.001	cardiolipin_synthase	T52_CON_SLT

wOo_09480	-5	0.001	lipoprotein_OsmY_ortholog	T52_CON_SLT
wOo_08030	-5	0.000	cytochrome_c-type_biogenesis_protein_CcmE	T52_CON_SLT
wOo_04000	-5	0.000	tRNA-Val-GAC	T52_CON_SLT
wOo_00150	-5	0.000	ABC-type transport system involved in cytochrome c biogenesis permease component	T52_CON_SLT
wOo_10680	-5	0.000	ferredoxin	T52_CON_SLT
wOo_08620	-6	0.000	hypothetical_protein	T52_CON_SLT
wOo_06150	-2	0.005	DNA_recombination_protein_rmuC-like_protein	T8_CON_SLT
wOo_03780	-6	0.000	YggT_family_membrane_protein	T52_CON_SLT
wOo_10280	-6	0.000	preprotein_translocase_subunit_SecY	T52_CON_SLT
wOo_02950	2	0.003	geranylgeranyl_pyrophosphate_synthase	T8_CON_ADT
wOo_07060	-2	0.001	transketolase	T8_CON_SLT
wOo_03620	-6	0.000	3R--hydroxymyristoyl-ACP_dehydratase	T52_CON_SLT
wOo_07090	-2	0.008	cytochrome_c_biogenesis_factor	T8_CON_SLT
wOo_09420	-3	0.001	permease	T8_CON_SLT
wOo_01620	-3	0.007	50S_ribosomal_protein_L10	T8_CON_SLT
wOo_04620	-6	0.000	lipoprotein_signal_peptidase	T52_CON_SLT
wOo_01810	-3	0.010	putative_monovalent_cationH+_antiporter_subunit_C	T8_CON_SLT
wOo_10500	-6	0.001	30S_ribosomal_protein_S10	T8_SLT_ADT
wOo_06060	-6	0.000	hypothetical_protein	T52_CON_SLT
wOo_08240	-3	0.008	prenyltransferase	T8_CON_SLT
wOo_01020	-3	0.001	phosphatidylglycerophosphatase_A_PgpA	T8_CON_SLT
wOo_03860	-6	0.000	putative_translation_factor_SUA5	T52_CON_SLT
wOo_03520	-4	0.008	amino_acid_transporter	T8_CON_SLT
wOo_09930	-6	0.000	hypothetical_protein	T52_CON_SLT
wOo_08680	-6	0.000	hypothetical_protein	T52_CON_SLT
wOo_03760	-7	0.000	tRNA_pseudouridine_synthase_A	T52_CON_SLT
wOo_05560	-6	0.001	RimM_protein_required_for_16S_rRNA_processing	T8_SLT_ADT

Appendix 6-3. *Onchocerca ochengi* Regulated Transcripts from Onchocercomata. Longitudinal differential expressions

GENE ID	log_2 FC	P-value	Annotation	Comparison
nOo.2.0.1.t12597-RA	10	0.000	antigen maltose binding protein	ADT_T48
nOo.2.0.1.t12681-RA	10	0.000	collagen alpha-1 chain-like	ADT_T48
nOo.2.0.1.t13501-RA	10	0.000	no description	ADT_T48
nOo.2.0.1.t08607-RA	9	0.000	no description	ADT_T48
nOo.2.0.1.t10492-RA	9	0.000	no description	ADT_T48
nOo.2.0.1.t10494-RA	9	0.000	#N/A	ADT_T48
nOo.2.0.1.t13462-RA	9	0.000	no description	ADT_T48
nOo.2.0.1.t11104-RA	9	0.000	no description	ADT_T48
nOo.2.0.1.t11709-RA	8	0.000	cre-tag-297 protein	ADT_T48
nOo.2.0.1.t13865-RA	8	0.000	amidophosphoribosyltransferase [Callosobruchus chinensis]	ADT_T48
nOo.2.0.1.t10208-RA	8	0.000	nematode cuticle collagen n-terminal domain containing protein	ADT_T48
nOo.2.0.1.t09695-RA	8	0.000	domon domain containing protein	ADT_T48
nOo.2.0.1.t12513-RA	8	0.000	no description	ADT_T48
nOo.2.0.1.t11532-RA	7	0.000	no description	ADT_T48
nOo.2.0.1.t09373-RA	7	0.000	cbn-mltn-7 protein	ADT_T48
nOo.2.0.1.t04360-RA	7	0.000	no description	ADT_T48
nOo.2.0.1.t13188-RA	7	0.004	#N/A	ADT_T48
nOo.2.0.1.t13067-RA	7	0.000	no description	ADT_T48
nOo.2.0.1.t13520-RA	7	0.001	no description	ADT_T48
nOo.2.0.1.t11152-RA	7	0.000	gcc2 and gcc3	ADT_T48
nOo.2.0.1.t03608-RA	7	0.000	astacin metalloprotease	ADT_T48
nOo.2.0.1.t01988-RA	7	0.000	no description	ADT_T48
nOo.2.0.1.t00277-RA	7	0.000	no description	ADT_T48
nOo.2.0.1.t11131-RA	7	0.000	no description	ADT_T48
nOo.2.0.1.t10608-RA	7	0.000	no description	ADT_T48
nOo.2.0.1.t06520-RA	7	0.000	hepatic leukemia factor	ADT_T48
nOo.2.0.1.t13449-RA	7	0.002	no description	ADT_T48
nOo.2.0.1.t09027-RA	7	0.000	heat shock protein 60	ADT_T48
nOo.2.0.1.t09055-RA	7	0.001	butyrate response factor 2	ADT_T48
nOo.2.0.1.t07588-RA	7	0.000	cre-tag-297 protein	ADT_T48
nOo.2.0.1.t06269-RA	7	0.001	cell death specification protein 2	ADT_T48
nOo.2.0.1.t11206-RA	7	0.001	no description	ADT_T48
nOo.2.0.1.t11443-RA	7	0.000	no description	ADT_T48
nOo.2.0.1.t12551-RA	7	0.001	no description	ADT_T48
nOo.2.0.1.t10815-RA	7	0.000	no description	ADT_T48
nOo.2.0.1.t10871-RA	6	0.001	no description	ADT_T48
nOo.2.0.1.t12435-RA	6	0.001	no description	ADT_T48
nOo.2.0.1.t06558-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t04545-RA	6	0.001	zinc finger	ADT_T48
nOo.2.0.1.t00085-RA	6	0.002	no description	ADT_T48
nOo.2.0.1.t12665-RA	6	0.000	estrogen receptor beta	ADT_T48
nOo.2.0.1.t10019-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t05947-RA	6	0.001	g2 mitotic-specific cyclin-b3	ADT_T48
nOo.2.0.1.t05200-RA	6	0.000	small family member (sma-9)	ADT_T48

nOo.2.0.1.t11374-RA	6	0.000	glutamate dehydrogenase	ADT_T48
nOo.2.0.1.t04544-RA	6	0.001	nucleolar gtp-binding protein 1	ADT_T48
nOo.2.0.1.t11335-RA	6	0.001	no description	ADT_T48
nOo.2.0.1.t05201-RA	6	0.000	atp-dependent clp protease atp-binding subunit clpx-mitochondrial isoform 2	ADT_T48
nOo.2.0.1.t08487-RA	6	0.001	no description	ADT_T48
nOo.2.0.1.t12987-RA	6	0.000	---NA---	ADT_T48
nOo.2.0.1.t06624-RA	6	0.000	hypothetical protein TRIADDRAFT_62725 [Trichoplax adhaerens]	ADT_T48
nOo.2.0.1.t12025-RA	6	0.000	pao retrotransposon peptidase family protein	ADT_T48
nOo.2.0.1.t02242-RA	6	0.000	cre-lpr-5 protein	ADT_T48
nOo.2.0.1.t09413-RA	6	0.002	prolyl 4-hydroxylase	ADT_T48
nOo.2.0.1.t06604-RA	6	0.001	no description	ADT_T48
nOo.2.0.1.t09889-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t07897-RA	6	0.000	protein isoform a	ADT_T48
nOo.2.0.1.t05418-RA	6	0.001	cre-col-109 protein	ADT_T48
nOo.2.0.1.t09546-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t04416-RA	6	0.001	fork head domain transcription factor slp2	ADT_T48
nOo.2.0.1.t04231-RA	6	0.000	atp synthase beta subunit	ADT_T48
nOo.2.0.1.t06625-RA	6	0.000	uvrabc system protein a- partial	ADT_T48
nOo.2.0.1.t04497-RA	6	0.002	no description	ADT_T48
nOo.2.0.1.t10267-RA	6	0.001	target of poxn	ADT_T48
nOo.2.0.1.t10476-RA	6	0.002	glutamate receptor 1	ADT_T48
nOo.2.0.1.t05102-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t06577-RA	6	0.001	variable abnormal morphology family member (vab-3)	ADT_T48
nOo.2.0.1.t11234-RA	6	0.001	deleted in malignant brain tumors 1 protein	ADT_T48
nOo.2.0.1.t13699-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t05047-RA	6	0.002	no description	ADT_T48
nOo.2.0.1.t06603-RA	6	0.002	twik (kcnk-like) family of potassium alpha subunit 39a	ADT_T48
nOo.2.0.1.t01172-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t10525-RA	6	0.000	leucine aminopeptidase-like protein	ADT_T48
nOo.2.0.1.t08770-RA	6	0.000	succinyl- ligase	ADT_T48
nOo.2.0.1.t10703-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t02122-RA	6	0.000	cre-wrt-9 protein	ADT_T48
nOo.2.0.1.t09210-RA	6	0.002	temporarily assigned gene name family member (tag-178)	ADT_T48
nOo.2.0.1.t10989-RA	6	0.003	bardet-biedl syndrome 2 protein homolog	ADT_T48
nOo.2.0.1.t09522-RA	6	0.004	cuticlin 1	ADT_T48
nOo.2.0.1.t11110-RA	6	0.003	cre-hhat-1 protein	ADT_T48
nOo.2.0.1.t02515-RA	6	0.003	transcription factor coe1	ADT_T48
nOo.2.0.1.t01103-RA	6	0.003	no description	ADT_T48
nOo.2.0.1.t02661-RA	6	0.000	phosphoribosylamine-glycine ligase	ADT_T48
nOo.2.0.1.t08089-RA	6	0.003	no description	ADT_T48
nOo.2.0.1.t09336-RA	6	0.003	hypothetical protein TRIADDRAFT_35139 [Trichoplax adhaerens]	ADT_T48
nOo.2.0.1.t10047-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t05255-RA	6	0.000	hypothetical protein TRIADDRAFT_62988 [Trichoplax adhaerens]	ADT_T48
nOo.2.0.1.t09663-RA	6	0.000	hepatic leukemia factor	ADT_T48
nOo.2.0.1.t12762-RA	6	0.003	ankyrin repeat and socs box protein 3	ADT_T48
nOo.2.0.1.t11546-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t04868-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t07515-RA	6	0.000	nadh dehydrogenase	ADT_T48

nOo.2.0.1.t10960-RA	6	0.004	no description	ADT_T48
nOo.2.0.1.t08283-RA	6	0.003	no description	ADT_T48
nOo.2.0.1.t07534-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t00955-RA	6	0.003	homeobox domain containing protein	ADT_T48
nOo.2.0.1.t07589-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t12566-RA	6	0.004	integrase core domain containing protein	ADT_T48
nOo.2.0.1.t06766-RA	6	0.004	no description	ADT_T48
nOo.2.0.1.t03924-RA	6	0.000	cytochrome c oxidase subunit i	ADT_T48
nOo.2.0.1.t07923-RA	6	0.000	protein isoform a	ADT_T48
nOo.2.0.1.t02241-RA	6	0.000	lpr-4 protein	ADT_T48
nOo.2.0.1.t10314-RA	6	0.004	cbr-eff-1 protein	ADT_T48
nOo.2.0.1.t12367-RA	6	0.004	heparan sulfate glucosamine 3-o-sulfotransferase 3a1	ADT_T48
nOo.2.0.1.t11578-RA	6	0.010	no description	ADT_T48
nOo.2.0.1.t03907-RA	6	0.004	no description	ADT_T48
nOo.2.0.1.t06897-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t07173-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t03747-RA	6	0.005	no description	ADT_T48
nOo.2.0.1.t01280-RA	6	0.005	pao retrotransposon peptidase family protein	ADT_T48
nOo.2.0.1.t11698-RA	6	0.005	metalloprotease 1 precursor	ADT_T48
nOo.2.0.1.t08002-RA	6	0.000	no description	ADT_T48
nOo.2.0.1.t10588-RA	6	0.000	sensor histidine kinase response regulator	ADT_T48
nOo.2.0.1.t13793-RA	5	0.000	dna mismatch repair protein	ADT_T48
nOo.2.0.1.t09251-RA	5	0.000	cuticlin 1	ADT_T48
nOo.2.0.1.t08001-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t12828-RA	5	0.000	low-density lipoprotein receptor domain class a containing protein	ADT_T48
nOo.2.0.1.t11749-RA	5	0.005	pan domain protein	ADT_T48
nOo.2.0.1.t08606-RA	5	0.005	no description	ADT_T48
nOo.2.0.1.t01374-RA	5	0.005	no description	ADT_T48
nOo.2.0.1.t04120-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t13136-RA	5	0.006	cre-mlt-10 protein	ADT_T48
nOo.2.0.1.t13438-RA	5	0.005	no description	ADT_T48
nOo.2.0.1.t04918-RA	5	0.007	no description	ADT_T48
nOo.2.0.1.t09827-RA	5	0.003	odr-3	ADT_T48
nOo.2.0.1.t08130-RA	5	0.000	kallmann syndrome 1 sequence	ADT_T48
nOo.2.0.1.t04164-RA	5	0.006	eukaryotic-type carbonic anhydrase family protein	ADT_T48
nOo.2.0.1.t04411-RA	5	0.003	myosin viia	ADT_T48
nOo.2.0.1.t12523-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t02255-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t02981-RA	5	0.000	cre-lgc-21 protein	ADT_T48
nOo.2.0.1.t06461-RA	5	0.001	leucine rich repeat family protein	ADT_T48
nOo.2.0.1.t07516-RA	5	0.000	deoxyribonuclease tatd	ADT_T48
nOo.2.0.1.t13452-RA	5	0.001	no description	ADT_T48
nOo.2.0.1.t03554-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t07451-RA	5	0.006	no description	ADT_T48
nOo.2.0.1.t12404-RA	5	0.006	helix-loop-helix dna-binding domain containing protein	ADT_T48
nOo.2.0.1.t05974-RA	5	0.008	no description	ADT_T48
nOo.2.0.1.t09038-RA	5	0.000	glutathione s-transferase domain-containing protein	ADT_T48
nOo.2.0.1.t06668-RA	5	0.000	nuclear hormone receptor family member nhr-25	ADT_T48
nOo.2.0.1.t04452-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t07379-RA	5	0.000	glutathione s- n-terminal domain containing protein	ADT_T48
nOo.2.0.1.t08642-RA	5	0.000	cre-col-182 protein	ADT_T48

nOo.2.0.1.t09565-RA	5	0.000	lectin c-type domain containing protein	ADT_T48
nOo.2.0.1.t10630-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t12692-RA	5	0.008	hypothetical kda protein in hem2-och1 intergenic	ADT_T48
nOo.2.0.1.t10526-RA	5	0.000	transcription termination factor rho	ADT_T48
nOo.2.0.1.t10779-RA	5	0.008	heparan sulfate glucosamine 3-o-sulfotransferase 3b1	ADT_T48
nOo.2.0.1.t02071-RA	5	0.001	no description	ADT_T48
nOo.2.0.1.t09146-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t02926-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t04498-RA	5	0.008	zinc c2h2 type family protein	ADT_T48
nOo.2.0.1.t13664-RA	5	0.008	no description	ADT_T48
nOo.2.0.1.t08342-RA	5	0.008	no description	ADT_T48
nOo.2.0.1.t13621-RA	5	0.008	no description	ADT_T48
nOo.2.0.1.t12798-RA	5	0.006	no description	ADT_T48
nOo.2.0.1.t12530-RA	5	0.005	nas-15 protein	ADT_T48
nOo.2.0.1.t07129-RA	5	0.004	acetyl-coa synthetase	ADT_T48
nOo.2.0.1.t09963-RA	5	0.001	no description	ADT_T48
nOo.2.0.1.t06078-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t08264-RA	5	0.009	no description	ADT_T48
nOo.2.0.1.t12369-RA	5	0.001	no description	ADT_T48
nOo.2.0.1.t08000-RA	5	0.000	---NA---	ADT_T48
nOo.2.0.1.t13363-RA	5	0.010	no description	ADT_T48
nOo.2.0.1.t03231-RA	5	0.002	fatty acid-binding protein	ADT_T48
nOo.2.0.1.t03675-RA	5	0.006	no description	ADT_T48
nOo.2.0.1.t10182-RA	5	0.002	nematode cuticle collagen n-terminal domain containing protein	ADT_T48
nOo.2.0.1.t01681-RA	5	0.001	pan domain-containing protein	ADT_T48
nOo.2.0.1.t13301-RA	5	0.002	discoidin domain-containing receptor 2-like	ADT_T48
nOo.2.0.1.t04364-RA	5	0.010	nematode cuticle collagen n-terminal domain containing protein	ADT_T48
nOo.2.0.1.t11269-RA	5	0.002	no description	ADT_T48
nOo.2.0.1.t13668-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t11272-RA	5	0.001	thyroid hormone receptor	ADT_T48
nOo.2.0.1.t04595-RA	5	0.000	lbp bpi cetp n-terminal domain containing protein	ADT_T48
nOo.2.0.1.t01389-RA	5	0.000	nuclear hormone receptor e75	ADT_T48
nOo.2.0.1.t12777-RA	5	0.007	integrase core domain containing protein	ADT_T48
nOo.2.0.1.t07294-RA	5	0.000	nematode cuticle collagen domain-containing protein	ADT_T48
nOo.2.0.1.t06331-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t09444-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t07380-RA	5	0.000	glutathione s-transferase domain-containing protein	ADT_T48
nOo.2.0.1.t04394-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t07517-RA	5	0.000	tyrosyl-trna synthetase 2	ADT_T48
nOo.2.0.1.t08769-RA	5	0.000	diaminopimelate epimerase-like	ADT_T48
nOo.2.0.1.t10589-RA	5	0.000	5 -nucleotidase sure-like	ADT_T48
nOo.2.0.1.t06262-RA	5	0.009	complexin precursor	ADT_T48
nOo.2.0.1.t00081-RA	5	0.004	vesicular glutamate transporter 3	ADT_T48
nOo.2.0.1.t09866-RA	5	0.003	no description	ADT_T48
nOo.2.0.1.t09042-RA	5	0.000	lbp bpi cetp c-terminal domain containing protein	ADT_T48
nOo.2.0.1.t08922-RA	5	0.003	no description	ADT_T48
nOo.2.0.1.t02676-RA	5	0.000	leucine rich repeat family protein	ADT_T48
nOo.2.0.1.t12049-RA	5	0.002	#N/A	ADT_T48
nOo.2.0.1.t08771-RA	5	0.004	fibronectin type iii domain containing protein	ADT_T48
nOo.2.0.1.t10766-RA	5	0.000	no description	ADT_T48

nOo.2.0.1.t11500-RA	5	0.002	no description	ADT_T48
nOo.2.0.1.t07744-RA	5	0.005	no description	ADT_T48
nOo.2.0.1.t08138-RA	5	0.000	hunchback transcription factor	ADT_T48
nOo.2.0.1.t08222-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t07546-RA	5	0.000	helix-loop-helix dna-binding domain-containing protein	ADT_T48
nOo.2.0.1.t09513-RA	5	0.000	no description	ADT_T48
nOo.2.0.1.t12555-RA	5	0.004	no description	ADT_T48
nOo.2.0.1.t08281-RA	5	0.000	caveolin 1	ADT_T48
nOo.2.0.1.t10210-RA	5	0.001	cuticle collagen dpy-2	ADT_T48
nOo.2.0.1.t12829-RA	5	0.001	cadherin domain-containing protein	ADT_T48
nOo.2.0.1.t06159-RA	5	0.000	period circadian protein	ADT_T48
nOo.2.0.1.t12915-RA	5	0.001	---NA---	ADT_T48
nOo.2.0.1.t08795-RA	5	0.000	cre-atf-2 protein	ADT_T48
nOo.2.0.1.t12429-RA	5	0.002	tyrosine-protein kinase src42a-like isoform 1	ADT_T48
nOo.2.0.1.t01803-RA	5	0.005	CES-1 [Brugia malayi]	ADT_T48
nOo.2.0.1.t10544-RA	5	0.001	cuticlin 1	ADT_T48
nOo.2.0.1.t09594-RA	5	0.003	gcc2 and gcc3	ADT_T48
nOo.2.0.1.t03748-RA	5	0.002	no description	ADT_T48
nOo.2.0.1.t03923-RA	5	0.000	glyceraldehyde 3-phosphate dehydrogenase	ADT_T48
nOo.2.0.1.t07225-RA	5	0.001	wrt-10 protein	ADT_T48
nOo.2.0.1.t11988-RA	4	0.002	no description	ADT_T48
nOo.2.0.1.t00013-RA	4	0.000	pan domain protein	ADT_T48
nOo.2.0.1.t06387-RA	4	0.000	von willebrand factor type a domain containing protein	ADT_T48
nOo.2.0.1.t10789-RA	4	0.002	lethal family member (let-653)	ADT_T48
nOo.2.0.1.t07481-RA	4	0.000	upf0639 protein	ADT_T48
nOo.2.0.1.t07749-RA	4	0.000	no description	ADT_T48
nOo.2.0.1.t11713-RA	4	0.000	no description	ADT_T48
nOo.2.0.1.t10758-RA	4	0.005	ankyrin repeat and socs box protein 3	ADT_T48
nOo.2.0.1.t07243-RA	4	0.000	hypothetical protein Bm1_50630 [Brugia malayi]	ADT_T48
nOo.2.0.1.t01785-RA	4	0.001	no description	ADT_T48
nOo.2.0.1.t06440-RA	4	0.008	cre-unc-10 protein	ADT_T48
nOo.2.0.1.t12383-RA	4	0.003	no description	ADT_T48
nOo.2.0.1.t07536-RA	4	0.009	cre-mex-1 protein	ADT_T48
nOo.2.0.1.t04580-RA	4	0.004	paired box protein pax-6	ADT_T48
nOo.2.0.1.t06089-RA	4	0.004	u uitin family member (ubq-1)	ADT_T48
nOo.2.0.1.t08970-RA	4	0.009	no description	ADT_T48
nOo.2.0.1.t06773-RA	4	0.004	cuticlin 1	ADT_T48
nOo.2.0.1.t07514-RA	4	0.002	udp-n-acetylmuramoyl-tripeptide--d-alanyl-d-alanine partial	ADT_T48
nOo.2.0.1.t02624-RA	4	0.000	no description	ADT_T48
nOo.2.0.1.t12934-RA	4	0.002	#N/A	ADT_T48
nOo.2.0.1.t12860-RA	4	0.003	no description	ADT_T48
nOo.2.0.1.t02059-RA	4	0.006	no description	ADT_T48
nOo.2.0.1.t08117-RA	4	0.001	no description	ADT_T48
nOo.2.0.1.t00992-RA	4	0.007	unc-3	ADT_T48
nOo.2.0.1.t12823-RA	4	0.008	no description	ADT_T48
nOo.2.0.1.t02391-RA	4	0.007	low quality protein: hemicentin-1-like	ADT_T48
nOo.2.0.1.t02243-RA	4	0.001	no description	ADT_T48
nOo.2.0.1.t06469-RA	4	0.003	cadherin domain-containing protein	ADT_T48
nOo.2.0.1.t11880-RA	4	0.001	homeobox protein onecut	ADT_T48
nOo.2.0.1.t06395-RA	4	0.001	cuticlin 1	ADT_T48
nOo.2.0.1.t07689-RA	4	0.005	osmotic avoidance abnormal family member (osm-3)	ADT_T48
nOo.2.0.1.t13116-RA	4	0.004	no description	ADT_T48

nOo.2.0.1.t06378-RA	4	0.009	no description	ADT_T48
nOo.2.0.1.t03961-RA	4	0.001	leucyl-cystinyl aminopeptidase	ADT_T48
nOo.2.0.1.t08455-RA	4	0.007	no description	ADT_T48
nOo.2.0.1.t08976-RA	4	0.000	leucine-rich repeat neuronal protein 2-like	ADT_T48
nOo.2.0.1.t10339-RA	4	0.008	no description	ADT_T48
nOo.2.0.1.t11410-RA	4	0.007	no description	ADT_T48
nOo.2.0.1.t05821-RA	4	0.003	hypothetical tyrosinase-like protein in chromosome	ADT_T48
nOo.2.0.1.t04864-RA	4	0.002	no description	ADT_T48
nOo.2.0.1.t05034-RA	4	0.001	ground-like domain containing protein	ADT_T48
nOo.2.0.1.t07375-RA	4	0.004	variable abnormal morphology family member (vab-3)	ADT_T48
nOo.2.0.1.t07090-RA	4	0.000	no description	ADT_T48
nOo.2.0.1.t10979-RA	4	0.000	no description	ADT_T48
nOo.2.0.1.t08674-RA	4	0.007	cuticle collagen dpy-2	ADT_T48
nOo.2.0.1.t06667-RA	4	0.009	nuclear hormone receptor family member nhr-25	ADT_T48
nOo.2.0.1.t07043-RA	4	0.000	pr domain zinc finger protein 1	ADT_T48
nOo.2.0.1.t06230-RA	4	0.007	egl-46	ADT_T48
nOo.2.0.1.t05378-RA	4	0.009	cugbp elav family member 4	ADT_T48
nOo.2.0.1.t01838-RA	4	0.002	long-chain-fatty-acid-- ligase partial	ADT_T48
nOo.2.0.1.t08350-RA	4	0.000	innexin family protein	ADT_T48
nOo.2.0.1.t10613-RA	4	0.006	myosin-viia	ADT_T48
nOo.2.0.1.t12332-RA	4	0.004	no description	ADT_T48
nOo.2.0.1.t10377-RA	4	0.003	cre-unc-71 protein	ADT_T48
nOo.2.0.1.t05282-RA	4	0.000	twk-7 protein	ADT_T48
nOo.2.0.1.t07924-RA	4	0.003	calcium binding egf domain containing protein	ADT_T48
nOo.2.0.1.t10187-RA	4	0.004	gcc2 and gcc3	ADT_T48
nOo.2.0.1.t00808-RA	4	0.000	kunitz bovine pancreatic trypsin inhibitor domain containing protein	ADT_T48
nOo.2.0.1.t11983-RA	4	0.007	myosin viia	ADT_T48
nOo.2.0.1.t00148-RA	4	0.005	ras-related protein rab-28-like isoform 2	ADT_T48
nOo.2.0.1.t06704-RA	4	0.003	protein phosphatase 1h	ADT_T48
nOo.2.0.1.t04883-RA	4	0.001	hydroxysteroid dehydrogenase	ADT_T48
nOo.2.0.1.t00712-RA	4	0.001	no description	ADT_T48
nOo.2.0.1.t05354-RA	4	0.008	gcc2 and gcc3	ADT_T48
nOo.2.0.1.t11750-RA	4	0.009	cadherin domain containing protein	ADT_T48
nOo.2.0.1.t02834-RA	4	0.001	zinc finger protein	ADT_T48
nOo.2.0.1.t12206-RA	4	0.003	no description	ADT_T48
nOo.2.0.1.t04981-RA	4	0.003	no description	ADT_T48
nOo.2.0.1.t03667-RA	4	0.000	no description	ADT_T48
nOo.2.0.1.t07982-RA	4	0.006	cuticle collagen dpy-7	ADT_T48
nOo.2.0.1.t11220-RA	4	0.004	protein-l-isoaspartate(d-aspartate) o-methyltransferase	ADT_T48
nOo.2.0.1.t05827-RA	4	0.005	bone morphogenetic protein 3b	ADT_T48
nOo.2.0.1.t12110-RA	4	0.003	no description	ADT_T48
nOo.2.0.1.t09205-RA	4	0.006	no description	ADT_T48
nOo.2.0.1.t02857-RA	4	0.000	patched family protein	ADT_T48
nOo.2.0.1.t03726-RA	4	0.001	patched family protein	ADT_T48
nOo.2.0.1.t05713-RA	4	0.004	no description	ADT_T48

Appendix 6-4: Published Articles Obtained from and during PhD studies.

- Chapter 3 was published as follows:

Immunotherapy with mutated Onchocystatin fails to enhance the efficacy of a sub-lethal oxytetracycline regimen against *Onchocerca ochengi*.
Bah G S, Tanya V N, Makepeace B L.
Vet Parasitol. 2015 Aug 15; 212 (1-2): 25-34. doi: 10.1016/j.vetpar.2015.06.005. Epub 2015 Jun 12. PMID: 26100152.

- While working on my thesis, I contributed to the following related publications:

Stage-specific Proteomes from Onchocerca ochengi, Sister Species of the Human River Blindness Parasite, Uncover Adaptations to a Nodular Lifestyle.
Armstrong S D, Xia D, Bah G S, Krishna R, Ngangyung H F, LaCourse E J, McSorley H J, Kengne-Ouafo J A, Chounna-Ndongmo P W, Wanji S, Enyong P A, Taylor D W, Blaxter ML, Wastling JM, Tanya VN, Makepeace BL.
Mol Cell Proteomics. 2016 Aug; 15(8): 2554-75. doi: 10.1074/mcp.M115.055640. Epub 2016 May 25. PMID:27226403

Extracellular Onchocerca-derived small RNAs in host nodules and blood.
Quintana J F, Makepeace B L, Babayan S A, Ivens A, Pfarr K M, Blaxter M, Debrah A, Wanji S, Ngangyung H F, Bah G S, Tanya V N, Taylor D W, Hoerauf A, Buck A H.
Parasite Vectors. 2015 Jan 27; 8:58. doi: 10.1186/s13071-015-0656-1. PMID:25623184

Efficacy of three-week oxytetracycline or rifampin monotherapy compared with a combination regimen against the filarial nematode *Onchocerca ochengi*.
Bah G S, Ward E L, Srivastava A, Trees A J, Tanya V N, Makepeace B L.
Antimicrob Agents Chemother. 2014; 58(2): 801-10. doi: 10.1128/AAC.01995-13. Epub 2013 Nov 18. PMID:24247133

Analysis of gene expression from the *Wolbachia* genome of a filarial nematode supports both metabolic and defensive roles within the symbiosis.
Darby A C, Armstrong S D, Bah G S, Kaur G, Hughes M A, Kay S M, Koldkjær P, Rainbow L, Radford A D, Blaxter ML, Tanya V N, Trees A J, Cordaux R, Wastling J M, Makepeace B L.
Genome Res. 2012 Dec; 22(12): 2467-77. doi: 10.1101/gr.138420.112. Epub 2012 Aug 23. PMID: 22919073

A worm's best friend: recruitment of neutrophils by *Wolbachia* confounds eosinophil degranulation against the filarial nematode *Onchocerca ochengi*.
Hansen R D, Trees A J, Bah G S, Hetzel U, Martin C, Bain O, Tanya V N, Makepeace B L.
Proc Biol Sci. 2011 Aug 7; 278(1716): 2293-302. doi: 10.1098/rspb.2010.2367. Epub 2010 Dec 22. PMID: 21177682

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Achukwi M, D., Harnett, W., Enyong, P. and Renz, A. (2006) 'Successful vaccination against onchocerca ochengi infestation in cattle using live onchocerca volvulus infective larvae', *Parasite Immunology*, 29(3), 113-116, available: http://dx.doi.org/10.1111/j.1365-3024.2006.00917.x.

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